


STATE LIBRARY OF PENNSYLVANIA



3 0144 00328620 0

5
614.405
J826
v. 41





Digitized by the Internet Archive
in 2015

<https://archive.org/details/journalofinfecti41unse>

The
Journal of Infectious Diseases

Published by the John McCormick Institute for Infectious Diseases

EDITED BY
LUDVIG HEKTOEN AND EDWIN O. JORDAN

IN CONJUNCTION WITH
FRANK BILLINGS F. G. NOVY
H. GIDEON WELLS KARL F. MEYER

Volume 41
1927

Chicago, 1927

Composed and Printed by
American Medical Association Press
Chicago, Illinois, U. S. A.

A NEW AGAR-DYE DIFFERENTIAL MEDIUM FOR THE COLON-TYPHOID GROUP

WITH SPECIAL REFERENCE TO ITS USE IN WATER ANALYSIS

A. J. SALLE

From the Laboratories of the Bureau of Sanitary Engineering, California State Board of Health, and the Division of Bacteriology, University of California Medical School, Berkeley

Since the classical work of Endo¹ who employed basic fuchsin decolorized with sodium sulfite for the isolation of the colon organisms, many modifications of the original formula as well as mediums employing other dyes have been reported. Without a doubt, the medium is preferred in water works laboratories. Modifications of the original formula have been reported by Kendall and Walker,² Kendall and Day,³ Kinyoun and Deiter,⁴ Robinson and Rettger,⁵ and Levine⁶ adapting the medium to special investigations. Instead of incorporating lactose in the preparation other sugars have been used. Harding and Ostenberg⁷ recommended xylose and arabinose and were able to classify the organisms of the paratyphoid or intermediate group into definite subgroups. Wurtz⁸ prepared lactose agar containing the vegetable indicator litmus. Meyer⁹ increased the concentration of agar to 3% and found less diffusion of acid into the surrounding medium, and, therefore, a restriction of the spreading forms. Drigalski and Conradi¹⁰ added gentian violet to the medium of Wurtz. This preparation inhibits the growth of gram-positive organisms.

There has been one great objection to the use of the various fuchsin-sulphite mediums, namely, their instability in the presence of light and heat. The color gradually returns on standing even at ice box temperature and thus limits the usefulness of the preparation. This disadvantage has been overcome by the well known work of Holt-Harris and Teague¹¹ who made use of eosin and methylene blue. The medium has more recently been greatly simplified by Levine.¹²

Various other dyes, on account of their selective action, have been useful. Conradi¹³ combined picric acid and brilliant green. Krumwiede, Pratt and McWilliams¹⁴ also recommended brilliant green while Teague and Clurman^{15, 16}

Received for publication, Jan. 21, 1927.

¹ Centralbl. f. Bakteriöl., 1, O., 1903, 35, p. 109.

² J. M. Research, 1910, 23, p. 481.

³ Ibid., 1911, 25, p. 95.

⁴ Am. J. Pub. Health, 1912, 2, p. 979.

⁵ J. M. Research, 1916, 34, p. 363.

⁶ Abst. Bact., 1918, 2, p. 38.

⁷ J. Infect. Dis., 1912, 11, p. 109.

⁸ Arch. de Med. exper. et d'Anat. path., 1892, 4, p. 85.

⁹ J. Bact., 1917, 2, p. 237.

¹⁰ Ztschr. f. Hyg. u. Infektionskrankh., 1902, 39, p. 283.

¹¹ J. Infect. Dis., 1916, 18, p. 596.

¹² Ibid., 1918, 23, p. 43.

¹³ Centralbl. f. Bakteriöl., 1, Ref., 1908, 42, p. 47.

¹⁴ J. Infect. Dis., 1916, 18, p. 1.

¹⁵ Ibid., p. 647.

¹⁶ Ibid., p. 653.

226835

3 100

favor eosin brilliant green agar for the isolation of the typhoid organisms from stools. Meyer and Stickel¹⁷ have also come to the same conclusions, although they incorporated the dyes in a peptic digest agar. Malachite green either alone or in combination with other dyes has been added by Loeffler¹⁸ to a gelatine or to an agar¹⁹ medium. Eric and Amy Kindborg²⁰ found a combination of malachite green with acid fuchsin suitable for their work. Among the many other differential mediums recommended are the following: malachite green, oxgall and sodium sulphite agar of Padlewsky;²¹ china blue, malachite green agar of Bitter²² and Bongartz²³ and Morishima²⁴ devised a china blue, lead acetate agar in two layers. Glassner²⁵ employed china blue, metachrome yellow agar. Meyer and Stickel¹⁷ recommended eosin china blue agar when prepared with peptic or tryptic digests.

Bile or its salts are extensively used in mediums because of their marked inhibitory influence on organisms occurring in soil and air. MacConkey²⁶ employed bile salt agar containing neutral red. Rector²⁷ made use of dried bile instead of bile salts.

Guth²⁸ substituted alizarin for litmus in the medium of Wurtz⁸. Kristensen, Lester and Jurgens²⁹ used bromthymol blue for litmus in Drigalski-Conradi medium.¹⁰ Baker³⁰ advocated the substitution of bromthymol blue for litmus in routine laboratory work. Simmons³¹ added agar and bromthymol blue to Koser's citrate medium.³²

In water analytical work the bacteriologist is not so much interested in the differentiation between nonlactose and lactose fermenting types of gram-negative bacteria, but the distinction between fecal and non-fecal organisms. As a rule, the hydrogen ion concentrations and the gas ratios distinguish *B. coli* and *B. aerogenes*. Clark³³ determined electrometrically the final hydrogen ion concentration produced by a large number of strains of *B. coli*. A simple test has been suggested by Clark and Lubs in ordinary medium³⁴ and in a synthetic medium³⁵ which will distinguish the two main groups of the colon-aerogenes family. In the course of extensive routine studies of water in varying

¹⁷ Ibid., 1918, 23, p. 62.

¹⁸ Deutsch. med. Wchnschr., 1903, 36, p. 286.

¹⁹ Ibid., 1907, 39, p. 1581.

²⁰ Centralb. f. Bakteriöl., 1, O., 1908, 46, p. 554.

²¹ Ibid., 1908, 47, p. 540.

²² Ibid., 1911, 59, p. 469.

²³ Ibid., 1913, 71, p. 228.

²⁴ J. Bact., 1918, 3, p. 19.

²⁵ Centralb. f. Bakteriöl., 1, O., 1918, 80, p. 219.

²⁶ J. Hyg., 1908, 8, p. 322.

²⁷ Am. J. Pub. Health, 1913, 3, p. 154.

²⁸ Centralb. f. Bakteriöl., 1, O., 1909, 51, p. 190.

²⁹ Brit. J. Exper. Path., 1925, 6, p. 291.

³⁰ J. Bact., 1922, 7, p. 301.

³¹ J. Infect. Dis., 1926, 39, p. 209.

³² J. Bact., 1924, 9, p. 59.

³³ J. Biol. Chem., 1915, 22, p. 87.

³⁴ J. Infect. Dis., 1915, 17, p. 160.

³⁵ Abstr. Bact., 1917, 1, p. 29.

degrees of pollution it appeared of great advantage to possess a method which would differentiate on a solid plating medium the two significant types of the colon group. In order to be practical such a medium should allow the growth of large distinctive colonies preferably with a metallic lustre and definite color changes in the medium.

Experimental.—Over fifty different dyes and indicators were tested. Finally, erythrosin, methylene blue and bromcresol purple were found to fulfill the desired prerequisites. The combination not only gave a good metallic appearance to the colonies, but also a good contrast from

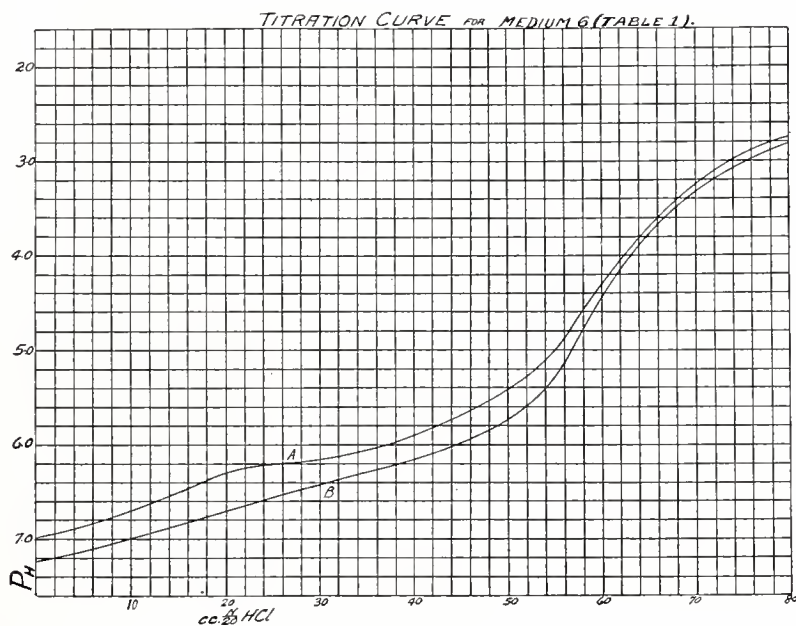


Fig. 1.—Titration curves for autoclaved (A) and unheated (B) mediums. P_H readings by hydrogen electrode, and titration figures in cc. of $\frac{N}{20}$ HCl for 100 cc. of medium, are used.

the background of the agar. Erythrosin is very similar to eosin chemically and in its appearance, but has the decided advantage over the latter, since it is nonfluorescent or only slightly so in aqueous solution. Methyl red is easily reduced by bacteria and, therefore, cannot be used.

Erythrosin, methylene blue and bromcresol purple change in color from purple to orange at about P_H 5.6. A medium, in order to give satisfactory results, must be so prepared that *B. coli* will grow and produce its maximum acidity at about P_H 5.0—5.4. This is only achieved by the addition of buffers. In table 1 the results of a preliminary test with six different kinds of broth containing increasing amounts

of buffer substances are summarized. The final hydrogen ion concentrations of the cultures were determined by means of the hydrogen ion electrode. All cultures were steamed in the Arnold to remove dissolved carbon dioxide and cooled to room temperature before making the readings. The tubes were incubated at 37 C. and the results recorded at the end of 24 hours. It is quite evident that as the amount of buffer is increased the final P_H of the medium is correspondingly increased.

TABLE 1
THE EFFECT OF AN INCREASE IN THE BUFFER CONTENT ON THE P_H OF THE MEDIUM.
1% Lactose Added to Each Formula, and Difco Peptone is Used

	1 Gm. Peptone 5 Water 1000 cc.	2 Gm. Peptone 10 Water 1000 cc.	3 Gm. Peptone 5 K ₂ HPO ₄ 2 KH ₂ PO ₄ 0.5 Water 1000 cc.	4 Gm. Peptone 15 Water 1000 cc.	5 Gm. Peptone 5 K ₂ HPO ₄ 5 KH ₂ PO ₄ 0.75 Water 1000 cc.	6 Gm. Peptone 5 K ₂ HPO ₄ 5 KH ₂ PO ₄ 1 Water 1000 cc.
Organ- ism Con- trol	P_H	P_H	P_H	P_H	P_H	P_H
	6.76	6.76	7.08	6.73	7.62	7.42
1	4.51	4.85	4.68	4.87	4.87	5.09
2	4.23	4.58	4.51	4.85	4.82	5.04
3	4.33	4.60	4.56	4.84	4.75	5.09
4	4.36	4.60	4.68	4.80	4.80	5.09
5	4.31	4.50	4.67	4.80	4.87	5.07
6	4.24	4.62	4.62	4.70	4.82	5.14
7	4.38	4.51	4.51	4.70	4.77	5.06
8	4.33	4.56	4.63	4.80	4.78	5.00
9	4.53	4.68	4.60	4.74	4.70	4.92
10	4.00	4.18	4.38	4.85	4.63	4.63
11	4.31	4.58	4.62	4.73	4.72	5.06
12	4.38	4.63	4.75	4.77	5.24	4.95
13	4.29	4.58	4.60	4.92	4.90	4.75
14	4.19	4.48	4.41	4.72	5.17	5.06
15	4.28	4.62	4.62	4.78	4.80	5.04
16	4.18	4.48	4.62	4.78	4.78	4.94
17	4.26	4.41	4.48	4.73	4.75	4.95
18	4.31	4.60	4.58	4.75	4.95	5.07
19	4.00	5.14	5.09	5.09	5.11
20	4.28	4.48	4.48	4.75	4.80	4.89
21	4.38	4.55	4.58	4.80	4.73	5.11
22	4.63	4.99	4.60	5.06	4.92	5.07
23	4.33	4.60	4.63	4.92	4.87	5.00
24	4.28	4.53	4.55	4.62	4.82	5.04
25	4.43	4.70	4.75	4.87	5.21	5.28
26	4.77	5.02	4.92	5.29	5.12

These results are in harmony with the statement by Clark ³⁶ that the greater the buffer value of the medium the smaller the final hydrogen ion concentration attained.

It is known ³⁷ that *B. aerogenes* will produce as much acid as *B. coli* if given sufficient fermentable carbohydrate. For a given amount of sugar *B. coli* will form more acid than *B. aerogenes*, provided the greatest amount added is just sufficient for *B. coli* to produce a final P_H of about 5.0. The amount of lactose that is just sufficient for *B. coli* to produce

³⁶ J. Infect. Dis., 1914, 14, p. 411.

³⁷ Ibid., 1915, 17, p. 160.

TABLE 2
FINAL HYDROGEN ION CONCENTRATION OF CULTURES OF B. COLI
Medium: Peptone, 5 gm.; K₂HPO₄, 5 gm.; KH₂PO₄, 1 gm.; water, 1000 cc.

Organism	pH in Different Mediums at Different Times					
	1% Lactose		0.5% Lactose		0.4% Lactose	
	24 Hours	48 Hours	24 Hours	48 Hours	24 Hours	48 Hours
1	5.09	4.85	5.12	5.04	5.36	5.43
2	5.04	4.92	5.33	5.22	5.51	5.60
3	5.00	4.48	5.19	5.00	5.29	5.39
4	5.09	4.87	5.11	4.94	5.29	5.44
5	5.07	4.89	5.12	4.99	5.58	5.70
6	5.14	4.92	5.31	5.09	5.66	5.90
7	5.06	4.90	5.31	4.92	5.22	5.39
8	5.00	4.87	5.31	5.60	5.40	5.51
10	4.63	4.68	4.70	5.06	4.89	4.90
11	5.06	4.89	5.16	5.00	5.63	5.95
12	4.95	4.77	5.09	5.17	5.48	5.78
14	5.06	4.75	5.16	5.11	4.94	4.90
15	5.04	4.94	5.22	5.75	5.36	5.46
16	4.94	4.75	5.09	4.90	5.19	5.41
17	4.95	4.85	5.19	5.14	5.70	6.78
18	5.07	4.85	5.16	4.99	5.33	5.38
20	4.89	4.78	5.19	4.84	5.51	5.71
21	5.11	4.94	5.19	4.94	5.31	5.41
22	5.07	4.67	5.43	5.09	5.78	6.32
23	5.00	4.89	5.07	4.97	5.68	5.65
25	5.28	4.85	5.53	5.90	5.87	6.98
26	5.12	5.16	5.69	5.51	5.88	6.26
24	5.04	5.02	5.66	4.94	5.46	5.51
27	5.09	4.90	5.43	5.16	5.51	5.41
28	4.94	4.85	5.19	5.09	5.34	5.36
29	5.06	4.89	5.24	5.06	5.73	5.75
30	5.04	4.82	5.11	5.11	5.51	5.77
31	5.12	4.92	5.12	5.07	5.36	5.61
32	5.28	5.07	5.22	5.26	5.55	6.17
33	5.06	4.84	5.17	4.97	5.29	5.51
34	5.04	4.89	5.12	5.12	5.44	5.61
35	4.95	4.85	5.04	5.29	5.41
36	5.04	4.89	5.12	5.16	5.34	5.39
37	5.11	4.94	5.31	5.29	5.61	5.73
38	5.04	4.85	5.28	5.11	5.34	5.39
39	5.04	4.87	5.21	5.44	5.38	5.63
40	5.00	4.85	5.22	4.99	5.80	5.88
41	4.92	4.77	5.02	4.84	5.36	5.53
42	4.99	4.89	5.24	4.97	5.58	5.65
43	5.02	4.89	5.21	5.04	5.24	5.34
44	5.04	4.87	5.22	5.02	5.41	5.51
45	5.14	4.90	5.16	5.16	5.68	5.83
46	4.78	4.65	4.87	4.95	4.87	5.02
47	5.06	4.89	5.34	5.02	5.77	5.90
48	5.12	4.95	5.26	5.17	5.68	5.66
49	5.28	4.90	5.22	5.02	5.58	5.78
50	5.00	4.85	5.17	5.02	5.26	5.46
51	4.77	4.77	4.85	4.89	5.36	5.61
52	5.24	4.99	5.11	5.02	5.31	5.33
53	5.00	4.87	5.07	5.02	5.63	5.66
54	5.02	4.92	5.36	5.14	5.53	5.88

its maximum acidity is inadequate for *B. aerogenes* to produce its limiting hydrogen ion concentration. In table 2 are shown the results obtained by inoculating lactose broth with cultures of *B. coli*, using 1%, 0.5% and 0.4% lactose, respectively. Formula 6 (table 1) was used. Readings were made at the end of 24 and 48 hours of incubation at 37 C. It is seen that as the amount of lactose is decreased the final hydrogen ion concentration is also decreased, until a concentration is reached ($0.4 \times$ lactose), which is insufficient for *B. coli* to produce its characteristic limiting P_H in the chosen medium. In order to sharply differen-

TABLE 3
FINAL P_H OF TWENTY-ONE STRAINS OF *B. AEROGENES*

Medium: Peptone, 5 gm.; K_2HPO_4 , 5 gm.; KH_2PO_4 , 1 gm.; water, 1000 cc.

Organism	0.6% Lactose		0.5% Lactose	
	24 Hours P_H	48 Hours P_H	24 Hours P_H	48 Hours P_H
Control	7.32	7.32	7.32	7.32
55	5.60	6.29	5.87	6.46
56	6.14	6.49	5.77	6.49
57	5.77	6.17	5.66	6.27
58	5.95	6.66	6.09	6.85
59	5.92	6.66	6.05	6.66
60	5.50	6.58	5.51	6.54
61	5.75	6.19	5.63	6.20
62	6.14	6.91	6.37	6.93
63	6.10	6.75	6.32	6.91
64	6.17	6.73	6.27	6.93
65	5.75	6.14	5.75	6.17
66	5.87	6.39	5.83	6.49
67	5.70	5.87	5.73	6.02
68	5.22	5.24	5.58	6.17
69	5.80	6.46	5.82	6.42
70	5.82	6.42	5.85	6.48
71	6.31	6.90	6.29	6.83
72	5.80	6.27	5.83	6.59
73	5.97	6.78	6.37	6.88
74	5.77	6.19	5.73	6.41
75	5.77	6.32	5.80	6.34

tiate *B. coli* from *B. aerogenes* the amount of lactose added must be just sufficient for *B. coli* to produce its limiting hydrogen ion concentration. An excess will permit *B. aerogenes* to produce the same P_H and the medium will be of little value. The results shown in table 2 suggested 0.5% lactose as the most suitable quantity.

The medium used for the tests recorded in table 2 and containing 0.5% and 0.6% lactose was inoculated with 21 strains of *B. aerogenes*. The final hydrogen ion concentrations were determined as before. The results are given in table 3. From these observations it became evident that the final P_H of the medium seeded with *B. aerogenes* is much higher than that inoculated with *B. coli*. A sharp differentiation between these two organisms is, therefore, readily possible.

The medium which was finally selected and which is now in use has the following composition:

Peptone (Difco)	5 gm.	Distilled water.....	1,000 cc.
K ₂ HPO ₄	5 gm.	Agar	20 gm.
KH ₂ PO ₄	1 gm.		

No adjustment of the reaction is required. This is controlled by the buffer salts. The ingredients are mixed and boiled until the agar is dissolved. The loss of water due to evaporation is restored. The agar is filtered while hot and distributed into 250 cc. Erlenmeyer flasks, 100 cc. to each flask. When ready to use, 0.5 gm. lactose is added to each flask and the agar melted in the Arnold or in boiling water. After the agar is melted the dyes are added to the contents of each flask: erythrosin (2% aqueous),* 2 cc., methylene blue (1% aqueous), 1 cc., and bromcresol purple (1% aqueous), 2 cc. The agar is rotated in the flask to obtain an uniform mixture and then poured into sterile petri dishes.

On this medium the colon organisms produce brilliant metallic colonies, slightly raised, with the surfaces usually flat or slightly concave. The surfaces are rarely convex. The colonies remain confined and show little tendency toward confluence. The agar surrounding the colonies will be changed in color from a purple to orange tinge. *B. aerogenes* strains rarely, if at all, produce metallic colonies and then only in the central portions. The colonies are raised, convex, very moist and show great tendency to run together. There is no change in the color of the agar. *Bacillus typhosus* does not ferment lactose and, therefore, produces no color changes. The growth appears as minute grayish colonies, on a purple background. The same is true of *B. paratyphosus* A and B, *B. enteritidis* and *B. dysenteriae*.

In figure 1 is shown a titration curve of medium 6 (table 1). One hundred cc. of the medium were titrated with $\frac{N}{20}$ HCl and the P_H readings followed by means of the hydrogen electrode. Curve A is for autoclaved, and curve B for unheated medium. Titration curves are very useful in that they tell at what P_H range the medium exerts its maximum buffer effect. They also help others to duplicate results. For instance, it makes no difference what brand of peptone is used so long as the titration curve is the same. From the chart it is evident tht it requires about one half as much acid to change the P_H of the medium from 6 to 5, as to change the P_H from 7 to 6. *B. aerogenes* produces its maximum acidity (using 0.5% lactose) at about P_H 6; *B. coli* has its limiting hydrogen ion concentration at P_H 5. Between P_H 6 and P_H 5 a relatively large change is obtained for a given amount of acid. This is

* The dyes used were obtained from the Coleman & Bell Co. Other brands may be substituted if the results are equally as good.

just what is desired, since such a medium will afford a sharp differentiation of the colon organisms. In table 4 are shown results obtained by streaking on the surface of the medium all strains of *B. coli* and *B. aerogenes* used in the previous tests. The results show perfect correlation.

The solid medium here described incorporates two tests in one operation. The value of such a differential medium is quite evident. In routine water examinations when speed is very essential, the agar dye preparation will shorten the period to complete an analysis by 24 hours. Instead of making one or more glucose broth seedings with typical colonies picked from Endo's medium or from eosin methylene blue agar, the distinction between a methyl red positive or negative strain may now be obtained on the plate at the same time. In the laboratories in which

TABLE 4
RESULTS OBTAINED BY STREAKING PURE CULTURES OF *B. COLI* AND *B. AEROGES*
ON THE DIFFERENTIAL MEDIUM

Organism	Number of Strains	Reaction on Agar		Correlation %
		Metallic Colony	Color	
<i>B. coli</i>	51	+	Orange	100
<i>B. aerogenes</i>	26	0	Purple	100

it is the custom to carry the test only to the partially confirmed stage, more satisfactory conclusions may now be drawn with the aid of this medium since two tests are better than one. Glucose broth tubes for the methyl red test will no longer be required, a saving in time and material.

SUMMARY

A new agar-dye differential medium for the identification of the members of the colon-aerogenes-typhoid group is described: containing peptone (Difco), 5 gm.; K_2HPO_4 , 5 gm.; KH_2PO_4 , 1 gm.; distilled water 1,000 cc.; agar, 20 gm.; lactose, 5 gm.; erythrosin (2% aqueous), 20 cc.; methylene blue (1% aqueous), 10 cc.; bromcresol purple (1%, aqueous), 20 cc., and by its use two tests are incorporated in one operation, thereby shortening the period of a complete water analysis by 24 hours. Glucose broth cultures may be dispensed with.

B. coli and *B. aerogenes* are sharply differentiated on this medium because of distinct differences in their carbohydrate metabolism.

THE RELATIONSHIP BETWEEN THE INTRACELLULAR GLOBULIN AND THE TOXIN OF CL. BOTULINUM

CASPER I. NELSON

From the Department of Hygiene and Bacteriology of the University of Chicago

A knowledge of the relationship borne by the vital protein constituents of a bacterial cell to its toxin would be of great assistance in determining the metabolic sources of the latter. A study of *C. botulinum* was recently undertaken with such an aim in mind. Results of previous work done by the author lead him to believe that in the specific intracellular globulin one would find a protein element vitally concerned in bacterial metabolism. For this reason the problem as stated has seemed to involve the questions: Are the toxins identical with the intracellular globulin; or is toxin a secretion produced from elements of the nutritive pabulum and related to the globulin only by association?

Methods.—Intraacellular globulins are obtainable from masses of bacteria by dialysis of the autolyzed masses. To eliminate the possibility of mixing globulins from the nutritive substances with those from bacterial sources it is necessary that synthetic mediums be used or that the organic sources of nitrogen be practically free of precipitable globulins. No synthetic medium has been devised as yet in which *Cl. botulinum* will grow luxuriantly enough to serve the purposes of this experiment. Ordinary nutrient peptones when tested give perceptible amounts of globulin-like precipitates. For this reason the following medium was used containing proteose peptone which is much freer of precipitable nitrogenous compounds than ordinary peptones:

Proteose peptone (Difco)....	10 gm.	Dextrose	10 gm.
Sodium chloride.....	5 gm.	Distilled water.....	1,000 cc.

The medium was adjusted to PH 7.4.

Two single cell strains of *Cl. botulinum* were chosen for the work, type A and type B, both isolated in this laboratory. Parallel tests were applied to both types of *Cl. botulinum* throughout the work. Cultures were prepared in 250 cc. Erlenmeyer flasks, each containing about 100 cc. of broth covered with a layer of sterile vaseline one inch thick. To obtain the best anaerobiosis the medium was inoculated as soon after sterilization as feasible while the vaseline was still liquid. For inoculation about 0.5 cc. of an old broth culture was transferred to each flask. All flasks were incubated on top of the incubator for six or seven days. The gas formed forced the vaseline up into the constricted portion of the flasks making a very tight seal. The growth was heavy. Type A *botulinum* was very susceptible to acid agglutination so that by the end of the incubation period most of the bacterial mass was precipitated. The acid agglutinability of

Received for publication, Feb. 23, 1927.

this type persisted throughout the experiment. Type B was not susceptible to acid agglutination and remained in suspension. The bacteria were concentrated by filtration and centrifugation. A concentrated mass of about 15 cc. was obtained from 10 flask cultures of each type. Only a very small portion of the mass consisted of spores.

Both cultures used are known to be toxigenic when they are grown in the appropriate veal medium for ten to fourteen days. But in the proteose broth very little toxin was produced in a week, at least 0.1 cc. being required to kill a white mouse. Globulin concentrates of several liters of the broth, however, were quite toxic, especially in the case of type B. Whenever the toxicity of a material was tested, both unprotected mice, and mice protected by excess injections of the anti-toxin, were used. No efforts were made to determine absolute titers, since the work was purely qualitative.

Although the culture broths were not very toxic the bacterial masses were very toxic. As little as .006 cc. of the massed cells killed mice within 24 hours. In this connection it should be noted that mice were killed in a shorter period of time than is usually considered normal for the action of botulinum toxin, but it did not seem to be atypical in any other respect since the appropriate antitoxin always protected. While some of the toxicity of the unwashed bacterial mass may have adhered to the cells from the broth, the low toxicity of the latter was insufficient to account for any but a small proportion of the total. In order to determine whether the toxin of the bacterial mass was extracellular or intracellular, the cells were washed repeatedly by suspending in cold distilled water (P_H 7.4) and centrifuging. Twelve to eighteen such washings failed to reduce the toxicity of the bacterial mass perceptibly, but each lot of wash water was somewhat toxic. The toxin in the cells was typically thermolabile, being destroyed by heating at 74° for 10 minutes. When the bacterial masses were freshly precipitated by centrifugation, and then suspended in thymolized water and placed in the refrigerator over night, the wash was still toxic. This was done on the assumption that possibly cell metabolism continued in the interims between washing and so elaborated more toxin. If such is the explanation thymol failed to inhibit toxin production. One effect of the repeated washing of the cells was the appearance of protein in the wash, indicating autolysis, exosmosis or protoplasmic solution. To avoid such loss of the cell contents the washing was not continued further.

Botulinum Globulin.—The bacterial masses were suspended in an equal volume of 1% sodium chloride solution and then autolyzed by

alternately freezing them in liquid air and thawing them by brief immersions of the container in boiling water. In this process the bacterial substances were exposed to a range of temperatures varying from -192°C . to about 25°C . Eight or ten such treatments evacuated most of the vegetative cells, leaving only a thin layer of ectoplasm within the cell walls. The spores were not affected. The cell debris was next removed by centrifugation. A clear straw colored fluid remained.* This was electrodialed as described elsewhere.¹ Within 48 hours a grayish precipitate was obtained which was biuret positive and was soluble in a weak alkaline solution. Electrodialysis of the toxic washes from the undialyzed cell masses gave a similar protein precipitate.

The bacterial autolysates just prior to dialysis were very toxic. After dialysis it was found that all of the toxicity had been concentrated in the precipitated globulins and that the autolysate fluid was no longer toxic. The globulin precipitated from the washes acted in a similar manner. Redissolving and redialyzing the globulins did not free them from toxin. The cell debris remaining after autolysis was toxic. This toxin, which eleven washings failed to remove, was thermolabile, was neutralized by the homologous antitoxin and was susceptible to decomposition by alkali strong enough to hydrolyze the associated bacterial protein. Alkaline hydrolysis took place between P_H 11 and P_H 12 in a few hours.

Since attempts to separate the toxic element from the globulin without destruction of the former were quite unsuccessful the process was reversed. A small quantity of the toxic globulin was suspended (undissolved) in sterile salt solution. To this was added a small quantity of fresh pepsin and HCl . This mixture was incubated at 37°C . over night. The globulin mass was completely dissolved. Apparently the toxicity of the solution was greater than before peptonization though this may have been due to the release of toxin from within the masses of globulin. The peptonized solution was not tested for the presence of unchanged globulin since it was clear that the globulin could not have remained in solution at the acidity of the digest (P_H 3). The toxin of the digested globulin solution still responded to the homologous type antitoxin.

* The autolysates of type A and type B strains of *Cl. botulinum* differed quite constantly in their P_H . Type A varied between P_H 7.3 and 7.4 while type B varied between P_H 7.0 and 7.1. Both were grown on the same medium.

¹ J. Infect. Dis., 1926, 38, p. 371.

DISCUSSION

It is possible to grow *C. botulinum* in a medium in which massive growth will take place without the appearance of much soluble toxin in the medium. In such a medium the toxin is located within the cells of the bacterial masses which are produced. Autolysis of these vegetative cells releases the toxin and it is found in intimate association with the bacterial globulin. Attempts to wash the toxin out of the vegetative cells showed that in proportion as the cells were autolyzed their intracellular globulin appeared and, in intimate association with it, the toxin. May it not be that a medium in which toxin diffusion is pronounced is one in which the progressive autolysis of the cells occurs? The usefulness of a medium as a nutritive base is not necessarily a measure of the usefulness of that medium in the production of soluble toxin. On the contrary it may be that more soluble toxin will appear the more cell mortality is increased by autolysis. Such an increase in soluble toxin should, in the light of the results of this research, be accompanied by a parallel increase in the growth of characteristic bacterial globulin that appears in the medium.

The method of autolysis used extracted the endoplasmic and cell sap portions of the cells. The ectoplasmic portion remained within the cell walls. Both the autolysate and the ectoplasmic portions carried toxin. The toxin lay chiefly in the portion most easily extracted by autolysis.

In spite of the intimate association between toxin and globulin it seems most probable that each has its own identity since digestion of the globulin by pepsin leaves the toxin at least unimpaired. Will this separation from its globulin shorten the stability of the toxin by a sort of starvation? It is a question quite worthy of further investigation.

CONCLUSION

The results appear to warrant the conclusions that the toxin of *C. botulinum* is elaborated within the cell in intimate association with the characteristic bacterial globulin; its appearance in the surrounding medium is associated with the globulin with which it is still bound, and is accompanied by cell mortality or disintegration, so that in this sense the toxin is not in itself a true secretion; and the toxin is evidently not identical with the intracellular globulin since it can be freed of the associated globulin by peptic digestion.

AN ACIDFAST ORGANISM ISOLATED FROM A MOUSE

MYCOBACTERIUM MURIS N. SP.

JAMES S. SIMMONS

From the Laboratory Service, Fitzsimons General Hospital, Denver, Colorado, and the Department of Laboratories, Army Medical School, Washington, D. C.

During an investigation made to determine the importance of wild gray mice as carriers of tubercle bacilli, an acidfast organism was isolated from the intestinal contents of one of the animals. One hundred mice, caught in the various wards and departments of a large hospital for tuberculous patients, were killed and examined for tubercle bacilli and pathologic lesions. Smears made of the gastric and intestinal contents, inguinal glands and various organs were stained by the Ziehl-Neelsen method and examined microscopically for acidfast organisms. Cultures of the gastric and intestinal contents were made, using Petroff's method. Stains and cultures were negative for tubercle bacilli, but an acidfast organism which so closely resembled the tubercle bacillus that it was at first mistaken for it, developed in cultures made from the intestinal contents of one mouse. The colonies of this organism which appeared after two weeks on Petroff's medium were small, flat, irregular in outline, dry and grayish white in color. Later they became heaped up, wrinkled and nodular. No pigment was produced in the primary cultures. The organisms from this culture were slender, nonmotile, gram-positive, acidfast rods which varied from 1.5 to 4, by 0.2 to 0.5 microns in size. Many of them had a banded and others a beaded appearance and the ends of some were slightly swollen giving them the appearance of the corynebacteria. The bacilli appeared singly and occasionally in pairs. No branching forms were seen. Neither flagella, capsules nor spores were demonstrable. In older cultures coccus like forms were seen.

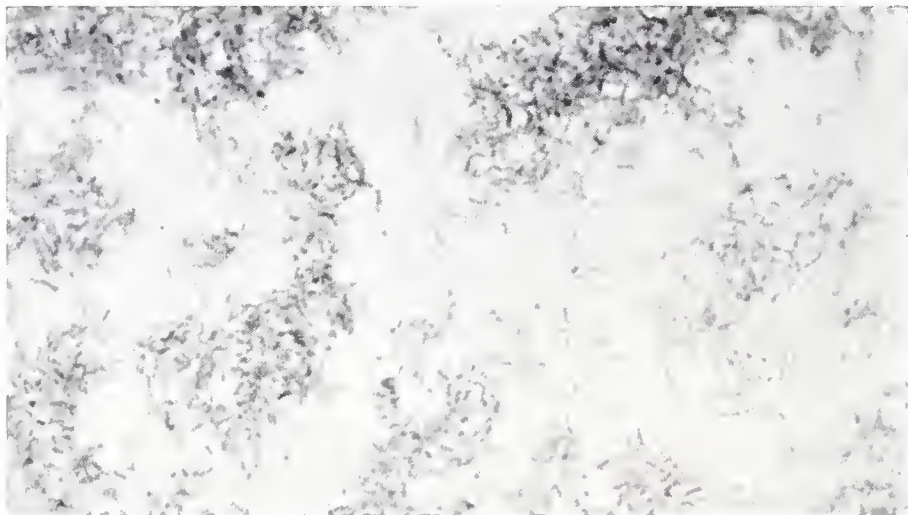
Cultural Characteristics.—When first isolated this organism grew only on culture mediums suitable for the development of tubercle bacilli, such as glycerol agar, glycerol broth, Petroff's medium, Dorsett's egg medium, etc.; and the colonies formed were similar to those produced by the human type of tubercle bacillus. However, since the cultural characteristics have changed somewhat, the growth obtained on different mediums will be considered separately.

Petroff's Medium: The organism was isolated on this medium and has grown luxuriantly in transfers made on it during the past three years. There

Received for publication, March 19, 1927.

has been very little change in the character of growth during this time. Small, grayish or yellowish white, dry, raised, irregular, wrinkled, crumpled colonies appeared in 12 to 14 days at 37 C. Growth was much slower at 25 C. or at 41 C. As the cultures became older the growth was more heaped up and nodular; and, as sometimes occurs in old cultures of tubercle bacilli, there was a slight reduction of the violet color of the medium. Transplants showed no pigment formation.

Glycerol Agar: The organism grew best at 37 C., and the colonies which appeared in about two weeks were quite like those formed by tubercle bacilli. They were small, grayish white, round, irregular, scaly, dry-looking. Later they become raised, more irregular in outline, confluent; and formed a rough, scaly, heaped-up, grayish white, nodular layer on the surface of the medium. No odor was noticed and the color was unchanged. The organisms did not grow except at the surface of the stab cultures. Later transfers on this medium, made after six months and after two years, were similar to the original except that the colonies had a light yellowish tan color.



Mycobacterium muris N. Sp., from culture grown for 1 month on Petroff's medium, and stained by the Ziehl-Neelsen method.

Coagulated Serum: Small white, round, scaly, dry colonies appeared after six to twelve days incubation at 37 C. These later became raised, irregular and heaped up. Transfers on this medium made six months and two years after the original isolation had a light yellowish color.

Coagulated Egg: The growth was similar to that on the coagulated serum medium except that in the transfers made two years after isolation the colonies were more round and discrete; and the yellow pigment was more pronounced.

Dorsett's Egg Medium: No pigment was produced in any of the cultures, all of which were either creamy white, or almost white; and the growth became heaped up and nodular quite like that on Petroff's medium.

Glycerol Broth: A granular growth occurred near the surface of the broth after two or three weeks. Pellicle formation was not noted. No striking color change occurred.

Glycerol Potato: Some growth occurred on glycerol potato medium immediately after the original isolation. This growth was dry, wrinkled, and

granular and was grayish tan in color. Cultures on potato after the organism had been transferred for over two years were similar in appearance with the exception that the color was a light yellowish tan.

On the following mediums no growth occurred when the culture was first isolated, but a slight growth was obtained after the original cultures had been transferred for two years: starch agar, few colonies, raised, irregular and white; Russell's double sugar medium, very few isolated, flat dry, grayish colonies which developed very poorly. No growth has been obtained either immediately after isolation or from cultures which had been transferred for 6 months or for 2 years, on 34 kinds of ordinary laboratory mediums: gelatin (surface or stab inoculation), nutrient agar (surface or stab inoculation), Endo's medium, lead acetate agar, lead acetate glycerol agar, nutrient agar or nutrient broth (containing lactose, glucose, sucrose, xylose, maltose, dextrin, levulose, mannitol, raffinose, galactose or inulin, 23 different combinations), nitrate agar, citrate agar, blood agar, nutrient broth, litmus milk, or Sabouraud's medium.

The organism was strictly anaerobic and grew best at 37 C. on all mediums used.

Pathogenicity.—This organism injected either subcutaneously or intraperitoneally was not pathogenic for white mice, guinea-pigs or rabbits. A salt solution suspension injected into the wattle of a hen produced a small local caseous lesion, without any other apparent change in the chicken's health. No other lesions were found when the chicken was killed and examined 6 months later.

SUMMARY

No tubercle bacilli were found in smears from the organs or gastrointestinal contents; or in cultures from the gastrointestinal contents of 100 wild gray mice caught in a hospital for tuberculous patients.

A related saprophytic, acidfast organism which was isolated from the intestinal contents of one mouse and observed for more than two years, was similar to *Mycobacterium tuberculosis* in morphology, staining reactions, slowness of growth, and requirements in mediums for growth. Transfers which have been carried on for two years, however, produced a yellowish tan pigment on glycerol agar. The organism was not pathogenic for white mice, guinea-pigs or rabbits and produced only a local lesion in a chicken.

In consideration of the characteristics and of the source of this organism (from *Mus musculus*) the name *Mycobacterium muris* is proposed.

SYNTHETIC MEDIUMS IN THE IDENTIFICATION OF THE TYPHOID-PARATYPHOID BACTERIA

LUTHER THOMPSON

From the Section on Clinical Pathology of the Mayo Clinic, Rochester, Minnesota

In spite of the various cultural and serologic tests used to identify members of the typhoid-paratyphoid group of bacteria, it is frequently impossible, in the laboratory, to establish their identity. In certain cases the fermentation of sugars is atypical, in others the agglutination test fails. Therefore any additional tests that may help in the separation of this group of bacteria should be useful.

It has been shown by Koser¹ that closely related types of gram-negative bacilli may be differentiated by the use of synthetic mediums. Up to the present time Kisch² seems to have made the most serious attempts to find synthetic mediums in which the typhoid, paratyphoid and dysentery bacilli would grow. He used a basic medium of inorganic salts to which was added 2% agar. As a source of nitrogen, various inorganic and organic nitrates and ammonium salts, and asparagin, urea and nucleic acid were added. Four of the six strains of *Eberthella typhi* grew on the mediums containing urea and asparagin. None of the compounds tried was satisfactory for growing *Salmonella paratyphi*, while nearly all were satisfactory for *Salmonella schottmülleri*. Pesch³ used a synthetic medium in which ammonium sulphate was the source of nitrogen, the carbon being added in the form of various organic acids. He found that *Salmonella schottmülleri* grew well in such a medium with both tartrate and citrate for sources of carbon, while *Eberthella typhi* and *Salmonella paratyphi* failed to grow. Recently Nelson⁴ used a synthetic medium containing ammonium succinate as a source of nitrogen, and he has grown a number of varieties of bacteria in the typhoid-paratyphoid-dysentery group in such amount that the growth could be used to prepare purified globulins from the various species of organisms. The work of Gordon and M'Leod,⁵ while not carried out with synthetic mediums, has shown that the amino acids can be grouped as favorable, inhibitory, or indifferent to some of the pathogenic cocci when added to an otherwise suitable medium. Braun and Calu-Bronner⁶ record the growth of *Salmonella schottmülleri* in a synthetic medium containing ammonium lactate as a source of nitrogen.

In the present work the following basic medium was used:

Na ₂ HPO ₄	1.0 Gm.	MgSO ₄	0.1 Gm.
KH ₂ PO ₄	1.0 Gm.	Glucose	2.0 Gm.
NaCl	5.0 Gm.	H ₂ O (distilled)	1,000 cc.

Received for publication, March 16, 1927.

¹ J. Bact., 1924, 9, p. 59.

² Centralbl. f. Bakteriöl., 1, O., 1919, 82, p. 28.

³ Ibid., 1921, 86, p. 97.

⁴ J. Infect. Dis., 1926, 38, p. 371.

⁵ J. Path. & Bact., 1926, 29, p. 13.

⁶ Centralbl. f. Bakteriöl., 1, O., 1921, 86, p. 1.

The various nitrogen-bearing compounds were added in 0.2% concentration. Besides the compounds listed in table 1 cystine and creatine were tried. The former was found to be affected by autoclaving, and if sterilized by filtering through a candle filter a precipitate formed on standing. The latter showed no advantage over some of the other compounds used. The medium was adjusted to a reaction of P_H 7.0 using bromthymol blue as indicator, and after tubing it was sterilized in the autoclave at from 12 to 15 pounds for fifteen minutes. It was found that the addition of bromthymol blue indicator (10 cc. of a 0.2% alcoholic solution for each liter) made the reading of the cultures easier since practically all of the organisms produced acid from glucose. However, the results obtained with the indicator were no different from those obtained without it. A few tests were made with mediums to which 1.5% agar had been added, but it was felt that the liquid medium was preferable, since slight amounts of peptone carried over with the inoculum from the stock agar slants would be less likely to give false results when diluted in a considerable volume of liquid than when smeared on the surface of a semisolid medium.

Most of the cultures used were obtained from various public health and commercial laboratories and were the ones used for producing immune serum or in making Widal tests. A few of the typhoid cultures were isolated in the course of routine work. All were run through the usual cultural tests including the action on 17 of the more common sugars, and agglutination tests were made on each culture against typhoid and the paratyphoid immune serums to check their identity. In this study 28 cultures of *Eberthella typhi*, 10 of *Salmonella paratyphi*, and 11 of *Salmonella schottmülleri* were tested. Four cultures of *Eberthella typhi* (Rawlings) obtained from widely separated sources, failed to ferment maltose and xylose. The agglutination test was less reliable than the sugar fermentations. On this group of cultures, ten of the typhoid cultures and four of the paratyphoid cultures giving inconclusive results on the first attempt. With these doubtful cultures, repeated attempts were made using immune serums from another source, and antigens made by transplant daily for eight days. The microscopic method of agglutination was used in order to note loss of motility, and a control test was made by the macroscopic method. In this way the doubtful agglutinations were made to give a fairly convincing reading except for one culture which was unagglutinable although the sugar fermentations were those of *Eberthella typhi*.

Table 1 shows the results obtained with the 49 cultures when various sources of nitrogen were used with the basic medium.

Stock cultures were kept on plain agar slants, and care was taken to use slants with no excess of moisture. Transplants were made about once a week to keep the cultures vigorous. In making the inoculations into the synthetic mediums, a straight wire was used which had been flattened at the tip. One side of this spatulate tip was touched lightly to the growth in the stock agar tube without coming in contact with the medium. The growth adhering to the wire was then carried into the tube to be inoculated. It was pressed against the glass below the level of the liquid until a faint smear of the organisms could be seen adhering to the inside of the tube. Incubation was carried on at 37 C., readings being taken after 1, 2, 3, 5 and 7 days. Seldom did any change occur after the third day. Growth was judged by turbidity, sediment and an acid reaction. In some cases in which growth was rather slow and scant the acidity was the best indication, although on shaking the tubes a small amount of sediment could be seen.

TABLE 1
RESULTS OF 49 CULTURES ON BASIC MEDIUM WITH VARIOUS SOURCES OF NITROGEN

Additions to Basic Medium		Positive Growth, %		
Compound	%	Typhoid	Paratyphoid A	Paratyphoid B
Potassium nitrate.....	0.2	0	10	45
Ammonium sulphate.....	0.2	10	20	100
Glycocoll.....	0.2	7	50	100
Alanine.....	0.2	10	40	100
Valine.....	0.2	3	10	100
Aspartic acid.....	0.2	18	50	100
Glutamic acid hydrochloride.....	0.2	18	50	100
Glutamic acid hydrochloride.....	0.5†	18	80	100
Glucose.....	0.5†			

From table 1 it will be seen that all cultures of *Salmonella schottmülleri* grew well in mediums made with nitrogen from several sources. Glutamic acid hydrochloride seemed to be about equal to aspartic acid, as might be expected from the similarity of their structure. In order to secure a medium on which more of the cultures would grow, tests were made with higher concentrations of glucose and glutamic acid hydrochloride. The results are shown in table 1. No doubt aspartic acid would have served as well as glutamic acid. By selecting a medium made with valine, and one made with the higher concentration of glutamic acid and glucose and comparing the results, it was seen that it was possible to separate the three organisms in a rather high percentage of cases. Of the strains of *Eberthella typhi*, 80% failed to grow in either medium. Of the strains of *Salmonella paratyphi*, 70% grew in the glutamic acid medium but not in the medium with valine. All strains of *Salmonella schottmülleri* grow well in both mediums. In order to compare related organisms and in general the gram-negative

bacilli which do not ferment lactose, the cultures listed in table 2 were tested on these two mediums. The two unnamed organisms in this table were isolated during routine work and represent fair examples of the problem of differentiating known pathogenic species and saprophytic intestinal forms. This species of *Eberthella* was nonmotile, did not form indol, and gave acid in glucose, galactose, xylose, and arabinose. None of the 12 other common sugars was affected. Positive growth in valine together with the sugar reactions made it improbable that it was a member of the dysentery group. The unnamed species of *Salmonella* gave sugar reactions much like those of *Salmonella paratyphi*, except for dextrin. The agglutination by the macroscopic method in paratyphoid A and B immune serums was entirely negative,

TABLE 2
COMPARATIVE RESULTS OF CULTURES GROWN ON VALINE AND GLUTAMIC ACID
Signs + and 0, indirect and no growth, respectively

Organism	Cultures	Growth on Mediums		Remarks
		Valine	Glutamic Acid	
<i>Eberthella paradysenteriae</i> (Hiss)....	3	0	Slight	Isolated from urine; resembles <i>Eberthella minutissima</i> (Kruse) Bergey, and others
<i>Eberthella paradysenteriae</i> (Flexner).	1	0	Slight	
<i>Eberthella</i> sp.?.....	1	+	+	
<i>Salmonella morgani</i>	3	0	+	Isolated from feces; sugar reactions like <i>Salmonella archibaldi</i> (Castellani and Chalmers)
<i>Salmonella enteritidis</i>	3	+	+	
<i>Salmonella</i> sp.?.....	1	+	+	
<i>Proteus ammoniae</i>	2	0	+	Clouding; no acid
<i>Proteus vulgaris</i>	1	0	+	
<i>Alcaligenes fecalis</i>	1	0	+	

yet in view of results previously obtained with the agglutination test, and considering the slight variation from the typical fermentations, it would not be possible to determine that the organism was not *Salmonella paratyphi* without more study. The fact that this organism grew in the valine medium was further indication that it was not *Salmonella paratyphi*. The use of synthetic mediums does not take the place of other tests, but seems to give valuable additional evidence in some of the doubtful cases.

SUMMARY

Forty-nine cultures of typhoid and paratyphoid bacteria were tested for their ability to grow in synthetic mediums to which various substances were added as the source of nitrogen. Valine and glutamic acid hydrochloride were selected as the most suitable among the compounds tried

for differentiation of members of this group. With valine, growth was obtained in 3% of the typhoid cultures, 10% of the paratyphoid A cultures, and 100% of the paratyphoid B cultures. With glutamic acid hydrochloride, the percentages of positive growth were, typhoid 18%, paratyphoid A 80%, and paratyphoid B 100%. Sixteen cultures of nonlactose-fermenting gram-negative bacilli, other than the typhoid and paratyphoid cultures, were tried on these two mediums for comparison. The reactions seemed specific for each species tried.

Synthetic mediums could be used to advantage as an additional cultural test in the identification of the bacteria studied.

PRECIPITIN, LYSIN AND AGGLUTININ TESTS WITH BILE

ETHEL B. PERRY

From the John McCormick Institute for Infectious Diseases, Chicago

Certain reactions of bile with toxins, blood cells, bacteria, and protein solutions simulate the antibody reactions of immune serums, and have been the object of many studies, the results of which may be briefly summarized. Antivenomous and other antitoxic properties of bile, in mixtures with the venom or toxin, are well established, but the protective value of bile injected separately is doubtful and the presence of "immunologic" antibodies therefore questioned. Cytolytic action on red blood cells and pneumococci, without the addition of complement, has been demonstrated for a great variety of biles. This cytolytic action, and the bacteriostatic or germicidal action demonstrated in most instances with typhoid or related organisms, have been attributed by the greater number of authors to the bile acids or salts, and to the higher unsaturated fatty acids, rather than to immune substances in the bile. These conclusions are generally based on the absence in the bile reactions of direct response to immunizing injections and of graded quantitative variations which are characteristic of serum reactions, on the inhibition by normal serums, and on the production of cytolysis and bacteriostasis with isolated acid or salt solutions. Nonspecific agglutinin for typhoid organisms and precipitin for egg white and dog serum have also been found in normal bile. Specific lysin and agglutinin have been found in the bile of immunized animals only after comparatively high titers have been attained in the serums, and perhaps more frequently are not demonstrable. Specific precipitin was not found in the one investigation reported. The detailed review of the literature follows.

Antivenomous and Antitoxic Action.—Frazer¹ states that injections into guinea-pigs, rabbits, and rats, of lethal doses of cobra and viper venoms mixed with small amounts of bile from poisonous and nonpoisonous snakes, from ox, guinea-pig or rabbit, were innocuous. There was also some evidence of curative effect with injections of unmixed bile 30 minutes after the venom was injected. Native snake remedies which were known to contain snake bile, were used in the wounds as well as internally, and native "doctors" resented inquiries for

Received for publication, March 29, 1927.

¹ Brit. M. J., 1897, 2, p. 125.

snake bile on the grounds that "they were getting to know too much." Wehrmann² also found that ox, eel and viper bile when mixed with eel serum or viper venom inhibited their toxic actions, but were not curative when injected separately. Since one of the toxins of snake venom is a hemolysin which perhaps is due to a lipolytic action,³ or activated by a lipoid,⁴ possibly the lecithin of the erythrocyte itself, an antihemolytic action of bile is indicated, and the close chemical relationship of bile acids, snake venom and cholesterol⁵ assumes special interest. Streptococcal hemolysis has been inhibited with bile and bile salts,^{5a} which did not, however, prevent the hemolytic action of saponin and oleic acid. Koch⁶ reported that bile from cattle that had died from plague, when injected into healthy animals produced a light immunizing sickness, and inferred that the bile contained a toxin or an attenuated form of the causative organism. Following this Kolle⁷ separated the corpuscular sediment of the bile which apparently contained organisms (destroyed by heat at 60 C., and by phenol 5%) capable of producing the disease, from the supernatant bile which would produce immunity but not the disease. Kolle did not think that any true antitoxin was present. Frantzius,⁸ on the other hand, working with bile from rabbits killed by rabies virus, thought that the inhibition of the virus in mixtures with bile and the protection afforded by separate injections of bile, indicated the presence of a true antitoxin. Normal ox, pig and sheep bile he said were inactive. Vallee⁹ agreed that mixtures of rabies virus and bile were inert, but found even normal bile thus "antiseptic" and did not observe any protection from separate injections. Vincent,¹⁰ in his study of the destruction of tetanus toxin in the digestive tract, states that mixtures of tetanus toxin with bile (of man, ox, dog, rabbit and guinea-pig) became atoxic in 15 to 20 minutes at 48 C., and in two hours at 16 to 18 C., and that 1 cc. of bile neutralized 20 to 50 lethal doses for guinea-pigs. Separate injections did not prevent or cure tetanus, though there was some slight delay, and he considered that possibly a thermolabile oxidase might be present to account for the toxin destruction.

Hemolytic Action.—Nolf¹¹ spoke of the hemolytic action of bile as a well known fact. He worked with ox bile and rabbit red-blood cells and considered the alkaline metal salts of the bile acids responsible, since the lytic action required less bile as the salt concentration was increased. Ruffer and Crendiropoulou¹² found the red cells of man, sheep, rabbit and guinea-pig susceptible to the bile of man, horse and rabbit, but with varying rapidity of lysis with different combinations. Rabbit liver extracts were not lytic* and normal rabbit serum was antihemolytic. The hemolytic property was adsorbed by charcoal, and might be an organic substance; and antiserum from rabbits after injections

* Recent work by Calvo-Criado¹³ states that of many tissue extracts the liver extract, according to spectrophotometer readings, was most destructive of hemoglobin. Human skin extracts were nearly as effective. The action of liver extracts was greatly augmented by the addition of spleen extracts. What may be relation of destruction of hemoglobin and laking of corpuscles (hemolysis) is not understood.

² Ann. de l'Inst. Pasteur, 1897, 11, p. 810.

³ Neuberg and Reichert; Biochem. Ztschr., 1908, 4, p. 281.

⁴ Kyes, P.: J. Infect. Dis., 1910, 7, p. 181.

⁵ Yonemura, S., and Fujihara, M.: J. Biochem., 1926, 6, p. 91.

^{5a} Gordon, J.: Brit. J. Exper. Path., 1927, 8, p. 38.

⁶ Koch, R.: Centralbl. f. Bakteriol., 1, O., 1897, 21, p. 256; also cited by Kolle.⁷

⁷ Kolle: Ztschr. f. Hyg. u. Infektionskrankh., 1899, 30, p. 33.

⁸ Centralbl. f. Bakteriol., 1, O., 1898, 23, p. 782.

⁹ Ann. de l'Inst. Pasteur, 1899, 13, p. 506.

¹⁰ Ibid., 1908, 22, p. 341.

¹¹ Ibid., 1900, 14, p. 656.

¹² J. Path. & Bact., 1903-4, 9, p. 278.

¹³ Biochem. Ztschr., 1925, 164, p. 61.

of dog bile, ("hemozotic serum") were more antihemolytic than normal serum. Two protein fractions of dog bile,¹⁴ neither of which was hemagglutinative or hemolytic, were both somewhat antihemolytic, and one slightly antiagglutinative (action against rabbit antiserum for dog blood). Rabbit antisera for one fraction (mucin) was strongly hemagglutinative (for dog corpuscles), and the antiserum for the other fraction (remaining proteins) was little agglutinative but slightly hemolytic. Antisera for whole bile were strongly agglutinative, doubtless because of the mucin fraction. The authors state that the hemolytic action of bile is chemical from the bile salts and not immunologic.

King and Stewart¹⁵ in a study of the toxic action of bile (known since the time of Majendie) which might be due to its hemolytic action, found the toxicity diminished by calcium lactate but restored again by treatment with hydrochloric acid, or heart muscle. Liebermann¹⁶ and Fenyvessy¹⁷ considered the action of bile salts as that of a hemolytic complement; and the possible influence of lipoids in the hemolytic action of bile has already been mentioned.^{3,4,5}

According to Kimura¹⁸ normal rabbit bile was hemolytic for sheep red blood cells, due to action of bile salts, but true hemolysins were not present in normal bile, and appeared in the bile of immunized rabbits only late (9 to 25 days after the last injection), and then were not comparable in amount to the hemolysin of serum. In passively immunized rabbits hemolysins appeared in bile within a few hours.

Bacteriolytic and Bacteriostatic Action.—Neufeld¹⁹ found that pneumococci were dissolved by bile (from man, dog, cat, and goat, rabbit) while many other bacteria were not. Nicolle and Abil-Bey²⁰ used beef bile which was only more convenient than rabbit bile and was not spoiled by sterilization. Sodium choleate was also effective. With reference to the reliability of the test in distinguishing pneumococci from streptococci Neufeld and Haendel²¹ and later Truche, Coton and Raphael²² state that all streptococci are insoluble in bile while all pneumococci are not soluble, and that there is a parallelism between solubility and virulence, the insoluble pneumococci being avirulent. In agreement with Neufeld many authors have attributed the solvent action to the bile salts, but Kelly and Gussin²³ who review the subject consider whole bile more effective than any of the separate factors. Kozlowski²⁴ found that the higher unsaturated fatty acids, in the form of soaps, in high dilutions were good solvents; Falk²⁵ has used sodium oleate; Atkins²⁶ in studying the action of sodium desoxycholate proposes that the reaction is merely an acceleration of normal autolysis, that the organisms from the papillae of autolysed colonies are not soluble. Serum prevents the solvent action (Avery, Chickering, Cole and Dochez.²⁷ The suggestion has been made that lowering the surface tension

¹⁴ Karsner, H. T., and Pearce, R. M.: J. M. Research, 1912, 26, p. 357.

¹⁵ J. Exper. Med., 1909, 11, p. 673.

¹⁶ Biochem. Ztschr., 1907, 5, p. 114.

¹⁷ Ibid., 1907, 4, p. 25.

¹⁸ Japan M. World, 1923, 3, p. 207.

¹⁹ Ztschr. f. Hyg. u. Infektionskrankh., 1900, 34, p. 454.

²⁰ Ann. de l'Inst. Pasteur, 1907, 21, p. 20.

²¹ Arb. k. Gsndtsamte., 1908, 28, p. 572.

²² Ann. de l'Inst. Pasteur, 1913, 27, p. 886.

²³ J. Infect. Dis., 1924, 35, p. 327.

²⁴ J. Exper. Med., 1925, 42, p. 453.

²⁵ J. Infect. Dis., 1926, 38, p. 8.

²⁶ Brit. J. Exper. Path., 1926, 7, p. 167.

²⁷ Monograph 7, Rockefeller Inst. for M. Res., 1917.

is the mechanism of lysis with bile but this does not invariably happen²⁸ and the addition of bile salts to serum or even bile may raise the surface tension probably by increasing the hydration of cholesterol which adsorbs the acid. Rosenthal²⁹ has stated that bile salts tend to free bilirubin (and other dyes excreted in the bile) from close union with the proteins of blood, thus sodium taurocholate would have great influence on diffusion and permeability—perhaps applicable to cells whether red cells or bacteria. In regard to the part played by the bile pigments Charrin and Rogers³⁰ thought bilirubin only slightly hemolytic and Sellards,³¹ who speaks of the marked cytolytic action of pigment exposed to light (photodynamic), demonstrated some bactericidal action but no appreciable lysis of pneumococci, found that alkalies affected solvency, and that the explanation of the solvent action of bile was far from complete. The bactericidal action of bile or bile salts studied by many, has been well reviewed by Neilson and Meyer³² who demonstrated also the bactericidal action of hepatic duct dog bile when it becomes alkaline on exposure to air, and the strong antiseptic action of taurocholic salts in alkaline medium. Fresh cystic ox bile is also germicidal on exposure to air while if protected by a layer of oil it is inactive. Bile (of rabbit and guinea-pig) and bile salts mixed with emulsions of infected liver destroy the virulence of *Spirochaeta icterohemorrhagiae*,^{32a} but a comparable effect from the jaundice of spirochetosis can hardly be assumed.

Bile from Immunized Animals: Vincent³³ states that complement-fixing bodies are not usually found in bile from rabbits immunized with typhoid bacteria; while Forster and Kayser³⁴ were occasionally able to demonstrate bactericidal properties for such bile when injected with the organism into the peritoneal cavity of guinea-pigs in the Pfeiffer bacteriolysin test. Neilson and Meyer³² tested the viability of typhoid organisms with fresh hepatic duct bile from immunized animals, found only an abrupt sterilization on the 5th day when exposed to air, an absence of graded diminution of action in quantitative dilutions, and other features at variance with the behavior of the corresponding serum, and from these concluded that bacteriolysins in the immunologic sense were not present in either normal or immune rabbit bile. Bacteriophagic lysis of typhoid organisms is inhibited by ox bile,³⁵ and upon this fact is based an explanation of typhoid carriers.

Agglutinins.—Tests for bacterial agglutinins in normal bile have apparently been made only for controls in other work and will be so included. Fodor and Rigler³⁶ claimed that in guinea-pigs one to two days after injections of typhoid organisms the bile contained traces of agglutinins which quantitatively were always inferior to the agglutinins of the serum. Ijischimpo³⁷ in studies of bile from convalescent typhoid patients found the titer of cystic bile much higher than that of hepatic duct bile (concentration of bile within bladder has often received comment), but far lower than the serum titer, and reactions in dilutions up to 1:20 must be considered nonspecific. Staubli³⁸ found traces of typhoid agglutinins in the bile of guinea-pigs and rabbits

²⁸ Adler, Solti, Hermer and Schmid: *Deutsch. med. Wchnschr.*, 1925, 51, p. 1689.

²⁹ *J. Pharmacol. & Exper. Therap.*, 1925, 25, p. 449.

³⁰ *Compt. rend. Soc. de biol.*, 1886, 3, p. 425.

³¹ *J. Am. M. A.*, 1918, 71, p. 1301.

³² *J. Infect. Dis.*, 1921, 28, p. 542.

^{32a} *Compt. rend. Soc. de biol.*, 1917, 80, p. 41.

³³ *Ann. de l'Inst. Pasteur*, 1912, 26, p. 381.

³⁴ *München. med. Wchnschr.*, 1905, 31, p. 1473; 52, p. 476.

³⁵ Calaub, G.: *Compt. rend. Soc. biol.*, 1925, 92, p. 1442. deJon, S. J., and Hauduroy, *Bull. de la Soc. méd. de hôp. de Paris*, 1925, 49, p. 1561.

³⁶ *Centralbl. f. Bakteriöl.*, 1, O., 1898, 23, p. 930.

³⁷ *Japan M. World*, 1925, 2, p. 102.

³⁸ *Centralbl. f. Bakteriöl.*, 1, O., 1903, 33, p. 375.

after immunization, and Cantani³⁹ similarly found agglutinins for colon and influenza bacilli. Venema,⁴⁰ Vincent and Fremiet,⁴¹ and Tsurumi and Kohda⁴² failed to find agglutinins in the bile no matter how high the titer of the serum. Neilson and Meyer³² reviewed the results of others, and in their own work found agglutinin for typhoid bacilli in normal rabbit bile 1:10, and in much higher dilutions in about 25% of the immunized rabbits, thus confirming the previous statement by Forster and Kayser.³⁴ In guinea-pigs agglutinin was not similarly demonstrated. In general the opinion was held that agglutinin was not secreted in the bile as in the milk.

Precipitins—Kimura³⁸ found normal rabbit bile strongly alkaline, but even when neutralized it produced precipitin reactions with dog serum and egg white. No specific precipitin was demonstrated in the bile.

No studies of bile in relation to anaphylaxis have been found but the action⁴³ of bile in preventing blood clotting in vitro by interference with the conversion of fibrinogen into fibrin suggests that bile, like heparin⁴⁴ might inhibit anaphylactic shock.

Precipitin Reactions with Rabbit and Dog Bile.—Gallbladder bile from rabbits killed after single injections of human hemoglobin⁴⁵ gave striking precipitin reactions not only with human hemoglobin but with other hemoglobin solutions (table 1), and reacted occasionally and to a lesser degree with normal rabbit serum. Also, bile from normal rabbits and from rabbits immunized with unrelated antigens reacted with the hemoglobin solutions, while bile from rabbits given single injections of beef lens solution practically never reacted with the lens solution. Cystic bile from 2 normal dogs did not react with these hemoglobin solutions, and fistular bile from dogs after one injection with rat or goat corpuscles (table 2) did not react with the homologous laked blood, except as noted, after storage for 25 to 30 days in the icebox. The fact that dogs do not produce precipitin serum may or may not be relevant.

The contact precipitin test was used, and readings made of the highest dilutions of the hemoglobin solutions which gave positive results at the end of one hour at room temperature. The reaction differed from that of immune serum in that the opacity was more diffuse, was usually above the initial plane of contact, and apparently was not formed in homogeneous mixtures of the bile and hemoglobin. Dilution of the bile with salt solution did not proportionately decrease the reaction, but bile mixed with an equal volume of normal rabbit serum was usually unreactive. Similar suppression of the lytic action of bile by nor-

³⁹ Compt. rend. Soc. de biol., 1886, 3, p. 425.

⁴⁰ Berlin. klin. Wchnschr., 1906, 43, p. 999.

⁴¹ Compt. rend. Soc. de biol., 1917, 80, n. 589.

⁴² Ztschr. f. Immunitätsforsch. u. exper. Therap., 1913, 19, p. 519.

⁴³ Haessler, H., and Stebbins, M. G.: J. Exper. Med., 1919, 29, p. 445.

⁴⁴ Kyes, P., and Strauser, E. R.: J. Immunol., 1926, 12, p. 419.

⁴⁵ Hektoen, L., and Schulhof, K.: J. Infect. Dis., 1922, 31, p. 32; 1923, 33, p. 224.

mal serum has been observed for pneumococci,³² and for red blood cells (with bile salts;⁴⁶ and the loss of virulence for spirochetes in contact with bile was less easily obtained with cultures in broth containing serum.^{32a} Bile which was unreactive when first removed from the gallbladder immediately after death would often react with a 1:200 or 1:400 dilution of hemoglobin after being kept 24 hours in the icebox. Irregularities in repeated tests were common, suggesting that

TABLE 1
PRECIPITIN REACTIONS OF RABBIT GALLBLADDER BILE WITH HEMOGLOBIN SOLUTIONS

Antigen Injected	Hours after Injection	Hemoglobins				
		Human	Horse	Sheep	Dog	Rabbit
Human hemoglobin.....	24	1600	1600	100	0	0
Defibrinated blood.....	48	12800	12800	400	1600	0
Hemoglobin.....	48	400	400	200	0	—
Citrated blood.....	72	6400	6400	800	0	0
Citrated blood.....	96	12800	0	0	0	—
Hemoglobin.....	96	800	0	0	—	0
Defibrinated blood.....	120	400	400	200	0	—
Hemoglobin.....	120	0	0	0	—	0
Hemoglobin.....	144	400	—	—	—	—
Horse pseudoglobulin.....	Varying stages of immun- ization	200	0	200	0	—
Sheep red blood cells.....		0	400	400	0	0
Ragweed pollen.....		3200	3200	800	800	0
Dog thyroglobulin.....		800	0	0	—	—
Toxin (<i>Streptococcus scarlatinae</i>)....		51000	6400	12800	800	0
		200	0	0	—	0
		1600	200	0	—	—
Streptococcus viridans.....		200	200	0	0	—
		1600+	800	0	0	0
		200	800	0	—	0
Antigen unknown.....(Long time after death)		25600	6400	6400	3200	1600
Controls.....	Normal	0(200)*	—	—	—	—
		0	1600	0	0	0
		6400	6400	0	0	0
		1600	—	—	200	—

Other controls: with normal rabbit serum irregular positive reactions were obtained; and with beef lens solution the bile of rabbits given injections of lens solution did not react.

* Bile was negative (0) when tested at once but positive after standing in icebox 24 hours. Such variations frequently occurred.

Figures indicate highest reacting dilution of hemoglobin; 0, no reaction; and dash (—), no test done.

the physical equilibrium of the bile was an important factor in the reaction. There was some evidence that the older solutions of hemoglobin also reacted more readily. The only reactions with dog bile were, as mentioned, with icebox specimens which had changed in color from amber to a deep green, suggesting that oxidative changes might have an influence. Changes in the germicidal power of bile exposed to air are already known.³² Dialysis acted much the same as dilution with salt solution in failing to produce proportionate reduction in the reaction.

⁴⁶ Donnelly, J. L., and Mitchell, A. G.: Am. J. Physiol., 1927, 79, p. 297.

The residue of the ether extract, redissolved in salt solution could not be used in precipitin tests because of cloudiness and low specific gravity but with washed erythrocytes produced marked disintegration and change of color from complete decolorization to green or greenish brown according to the dilution used. The increase in volume during dialysis was remarkable, being from 1 cc. to 140 cc. in one instance with dog bile, an interesting change in view of the concentrating action that takes place within the gallbladder.

TABLE 2

LYSIN, AGGLUTININ AND PRECIPITIN IN SERUM AND BILE FROM DOGS AFTER SINGLE INJECTIONS OF RED BLOOD CELLS

1 cc. of 5% suspension per kilo, intravenously.

Antigen	Days after Injection	Blood Serum			Bile from Fistula		
		Lysin	Agglutinin	Precipitin	Lysin	Agglutinin	Precipitin
Goat corpuscles	(Before injection)	24	0	0	3	0	0
	1 and 2	24	0	0	3	0	0
	3	48	0	0	3	0	0
	4	96	3	0	3	0	0
	5	192	3	0	3	0	0
	6	384	12	0	3	0	0
	7	768	96	0	3	0	0
	9	1536	768	0	3	0	0
	11	1536	1536	0	3	0	0
	13	3076	768	0	3	0	0
	16	1536	192	0	(3-96)*	48*	0
					(slight)		
	19	768	96	0	3	0	0
Rat corpuscles	(Before injection)	0	48	0	12	0	0
	1	0	48	0	12	0	0
	2	0	96	0	12	0	0
	3 (66 hours)	0	192	0			
	4	0	384	0	12	0	0
	5	0	384	0	12	0	0
	7	12	768	0	12	0	0
	11	48	768	0	12	(48 only)*	0
	13	192	1536	0	12	0	0
	17	3	768	0	12	0	0

* Irregular results might be due to contamination of bile from serum oozing about fistula.

Because of the close physiologic and chemical relationship between hemoglobin and at least certain constituents of bile these reactions easily attract even greater attention than do other nonspecific precipitin reactions of bile, but in the light of behavior which in so many respects is unlike that of immune serum they can hardly be interpreted as evidence of a true antibody response in the bile.

Hemolysin and Agglutinin Reactions with Fistular Dog Bile.—Simple fistulas, produced without evident injury to the common bile duct by suturing the edges of the incised gallbladder to the edges of the incision through the abdominal wall, allowed the collection of freshly

secreted bile throughout a period of about three weeks. A single injection of a 5% suspension of washed red blood cells, 1 cc. per kilo, was made intravenously. Goat and rat cells were used respectively for the two dogs. The bile and serum from each dog were tested on the day of collection. The tests consisted of the addition of 0.2 cc. of a 2% suspension of the antigenic cells (containing 0.0125 cc. fresh guinea-pig serum for the lysin test), to 0.4 cc. of each dilution of the bile or serum, with readings made after incubation at 37 C. for 1 hour. The highest reacting dilutions of the serum or bile were recorded (table 2). The reactions produced with normal bile in low dilutions remained unaffected by the injection of blood cells which produced typical antibody responses in the serum. The possibility that antibodies such as are demonstrated in serums might be obscured by the peculiar character of bile was considered, but a serum of high titer (1:1,536 lysin, 192 agglutinin for goat cells) mixed with an equal amount of bile collected on the same day, reacted without change of titer in either lysin or agglutinin test. The addition to bile of an equal volume of normal serum should therefore allow the demonstration of antibodies in the bile, but not any were so detected. Vincent and Fremiet⁴¹ also demonstrated that bile had no contrary action for the complement-fixing power of serum.

Perhaps with further immunization antibodies might have appeared in the bile, but so far as the experiment was carried the results support the findings of Venema, Vincent and Fremiet, and Tsurumi and Kohda that agglutinins are not found in the bile. Kimura¹⁸ found hemolysins only late after the last of several injections, and then in comparatively low dilutions.

CONCLUSIONS

Precipitin reactions of human hemoglobin with gallbladder bile from rabbits killed after single injections of human hemoglobin could not be interpreted as evidence of a true antibody response in the bile, because many nonspecific precipitin reactions were obtained with a variety of hemoglobin solutions and bile, and because the reactions with bile differ so essentially from those with immune serums. Neither normal nor specific precipitin was found in fresh dog bile from either gallbladder or fistula.

Fresh fistular bile from untreated dogs was not agglutinative, but was lytic (either with or without complement) for the red blood cells of goat and of rat. These reactions with bile occurred in low dilutions only and were unaffected by single injections of erythrocytes which produced characteristic antibody response in the serum.

THE PAUL TEST IN THE DIAGNOSIS OF SMALLPOX

JOHN A. TOOMEY AND JOHN A. GAMMEL

From the Department of Pediatrics, Western Reserve University and the Division of Contagious Diseases, Cleveland City Hospital, Cleveland, Ohio

In 1892, Guarnieri¹ showed that scarification of the rabbit cornea, followed by inoculation of the same, with the pustular contents of cowpox, or smallpox, caused an active proliferation of the epithelial cells within 24 to 48 hours. These lesions appeared as little hummocks, the center of which degenerated, leaving a crater-like depression. Within the cytoplasm of some of the affected cells were found curious inclusions, commonly spoken of as Guarnieri bodies—of variable size, ranging from a micron to about one-third the size of the nucleus of an epithelial cell; usually round, oval or lenticular, but not infrequently a little irregular in form. They stain with nuclear stains, but when a combination of eosin and hematoxylin is used, they present a mixed reaction in which the affinity for the acid dye seems to be more pronounced. Their parasitic nature is no longer accepted, but all workers in the field seem to agree that their presence is pathognomonic of variola.

Juergens² drew attention to the fact that the peculiar proliferation of the corneal epithelium which follows inoculation with smallpox material, may be observed in the living animal on examination of the cornea with a hand lens. One sees after 36 to 48 hours, transparent little hummocks which might be easily overlooked on direct examination.

Paul³ described a new technic to make these hummocks stand out macroscopically. He enucleated the inoculated eye after 48 hours or more, and placed it in sublimate alcohol, and after a few minutes' exposure, the lesions stood out prominently as intensely white elevations on a dull milky white background, with occasionally a distinctly visible, small dark crater in the center of the elevation. This was never seen after the inoculation of varicellar material and is very different from the picture presented by a purulent process in the cornea. The number of little elevations is variable. Sometimes, one sees only 3 or 4, while at other times, 20 and more may be discernible. Usually they are discrete

Received for publication, March 26, 1927.

¹ Quoted by Friedman, U., and Gins, H. A.: *Deutsch. med. Wchnschr.*, 1917, 37, p. 1159

² *Berlin. klin. Wchnschr.*, 1905, 42, p. 308.

³ *Wiener. klin. Wchnschr.*, 1916, 19, p. 996.

and circular in outline, with very clear margins. Occasionally, they show a tendency to become confluent.

Hoffman⁴ used the test for differentiation, and 90 to 95% of his cases gave positive reactions, but the total number of tests done was not mentioned. Loewenthal⁵ had 23% failures in 320 cases and recommends that the macroscopic diagnosis be read after 72 hours instead of 48. Scott and Simon⁶ investigated 18 cases of smallpox, 17 of which gave a macroscopic Paul reaction. Material from chickenpox vesicles, as well as material from the lesions of herpes febrilis, herpes zoster, herpes genitalis, dermatitis herpetiformis, pemphigus, and ringworm, gave negative tests as also did cultures of streptococci and staphylococci.

We tested this macroscopic reaction in the small epidemic in 1923, 1924, and 1925. Pustular material was rubbed into the cocainized and scarified cornea of the rabbit. After 48 hours the animal was killed,

TABLE 1
RESULTS OF THE PAUL TEST OF 80 PATIENTS WITH A DIAGNOSIS OF SMALLPOX

Examination of Pustules from	Number	Results of Tests on Rabbit Cornea		
		Positive	Negative	% Positive
Patients with smallpox.....	80*	45	35	56—
Control patients with simple scratching (30), chickenpox (10), impetigo (5), scarlet fever with vesiculopustular eruption (2), acne (9), and pustular syphilide (4).....	60	0	60	0

* Actual number of tests 101, included 21 tests repeated 2 or 3 times but with negative results although diagnosis was undoubtedly smallpox.

the eye removed and immersed in a sublimate bath for 10 minutes and then in alcohol for 5 to 10 minutes, after which it was examined with a hand lens. Simple pustular, as well as hemorrhagic types were examined, the results being tabulated in table 1.

Gins¹ was able to obtain positive Paul tests from the mucous membranes of the nose, but inferred that visible lesions must be presented before the test could be positive. A comparison between the reactions obtained from the nasal swabbings and pustules of the same patient may be obtained by referring to table 2.

One case of virulent or hemorrhagic smallpox gave a negative reaction with contents of the skin lesions. This occurred in a colored woman who had but a few small deeply buried petechiae. This patient was taken ill July 3rd, and died on July 5th. There were no gross lesions in the nose, nevertheless swabs from the nasal spaces produced a

⁴ M. Record, 1921, 100, p. 936.

⁵ Ztschr. f. Hyg. u. Infektionskr., 1924, 103, p. 722.

⁶ Am. J. Hyg., 1923, 3, p. 401.

violent general keratitis within 24 hours. One nasal swab was put in 5 cc. of saline and tested again. It, too, gave a violent reaction. Three cases, subsequently showing hemorrhagic lesions in the skin, gave positive Paul tests from the nasal washings before the rash appeared; in one case—2 days before.

That this lesion is due to the smallpox virus is evident from the following experiment. The pustular contents of several lesions was pooled, in each of 8 cases. One-third of the material was combined with an equal volume of convalescent smallpox serum and applied to the right eye of 8 rabbits. (This serum was obtained 30 days after the rash had appeared in a confluent variola vera.) One-third of the

TABLE 2
COMPARISON OF PAUL TESTS WITH NASAL SWABBINGS AND WITH PUSTULAR CONTENTS

Type Smallpox	Number of Cases	Nasal Swabbings				Pustular Contents			
		Positive		Negative	Doubtful	Positive		Negative	Doubtful
		No.	%			No.	%		
Severe, confluent pustular, hemorrhagic	15	11	76	2	2	14	93	1	0
Mild pustular	10	2	20	8	0	7	70	2	1

TABLE 3
PREVENTION OF KERATITIS WITH CONVALESCENT SERUM

Number of Patients		Pustular Content Alone	Pustular Content Plus Equal Parts Convalescent Smallpox Serum	Pustular Content Plus Equal Parts of Serum of Patient Never Vaccinated
8	Positive.....	8	0	8
	Negative.....	0	8	0

virus solution was untreated and applied to the left eye after scarification. The remaining third was combined with an equal volume of the serum of a patient who had never been vaccinated and applied to the eyes of a second series of rabbits. The results are contained in table 3.

In borderline cases, the reaction does not help much, as it is negative in too great a proportion of instances. It is nearly always positive in the pustular stage, especially in virulent cases, but in this stage there is usually no question as to the diagnosis.

CONCLUSION

A series of 80 cases of smallpox is reported, 45 of which gave positive Paul tests.

Paul's test when present, may be considered pathognomonic of smallpox.

THE PRODUCTION OF DIPHTHERIA ANTITOXIN

ARTHUR LOCKE, E. R. MAIN AND F. A. MILLER

From the Henry Baird Favill Laboratory of St. Luke's Hospital, Chicago

Preparations of diphtheria antitoxin differ widely in the rapidity with which they can neutralize a given preparation of diphtheria toxin. Since the more quickly acting or avid preparations may have a greater therapeutic effectiveness,¹ it has seemed worth while to undertake a study of antitoxin production with the aim of determining the factors which make for increased avidity.

The horses whose immunization is described in the accompanying curves and tables were selected from a series being used for commercial production by the Eli Lilly & Company biological laboratories. The spacing of the injections given eight of these horses is indicated along the horizontal margins of figure 1; the broken, vertical lines representing the quantity of antigen, in Lf units, injected upon the day indicated. The Lf unit was chosen as the unit most nearly measuring the antigenic capacity of the toxin.²

Samples of blood were drawn from the horses at regular intervals during the immunization, as indicated on the curves, and sent promptly and without alteration to this laboratory, where they were immediately titrated against a single preparation of mature toxin. Neither the titer nor the flocculation time of these samples was significantly affected by the conditions of transit. The inverse flocculation time of these samples, that is, the unit one divided by the flocculation time as observed in hours, is indicated, in figure 1, by squares connected by a broken line and referred to the inside numerals at the left of the page. The observed titers are indicated by circles, connected by a solid line and referred to the numerals along the left margin.

It is to be noted that the titer of the plasma of every horse has increased smoothly and without interruption so long as the associated inverse flocculation time has held constant or decreased. Wherever the inverse flocculation time has increased, the rate of increase in the titer has been definitely retarded. This relation should prove to be of

Received for publication, March 29, 1927.

¹ Locke and Main: *J. Infect. Dis.*, 1926, 39, p. 484.

² Ramon: *Ann. de l'Inst. Pasteur*, 1924, 38, p. 1.

some usefulness in practical antitoxin production since it may offer a convenient index as to how successfully immunization is proceeding. The status of the immunization may be determined in very little time by drawing a 15 to 20 cc. sample of blood, with precaution against coagulation, separating the plasma by centrifugation, and making a triplicate titration as follows:

Graded volumes of a dilution of the plasma, not exceeding 0.1 cc. in quantity, are measured into the bottoms of a series of 8x75 mm. test-tubes with the aid of a calibrated micropipet which is graduated in

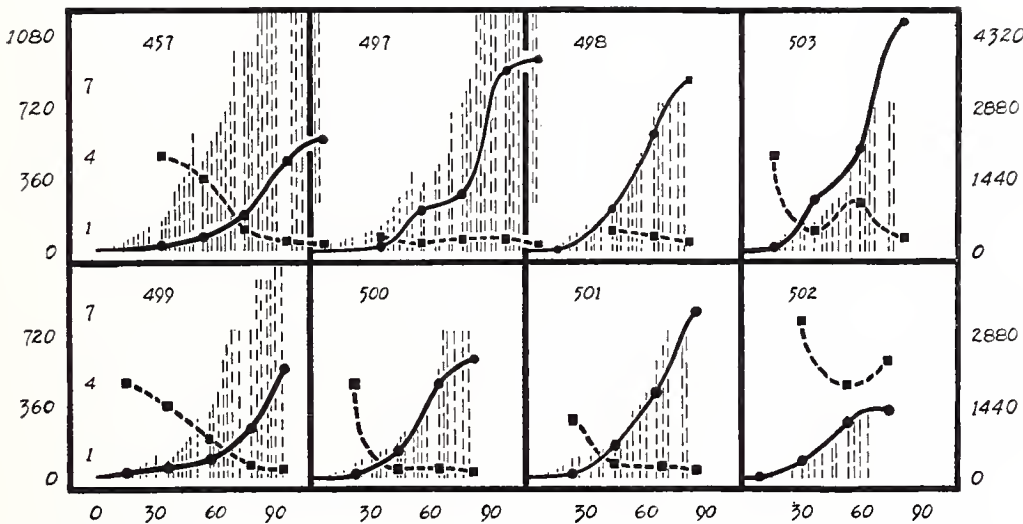


Fig. 1.—The relation between the size and spacing of the antigenic stimulus and the titer and flocculation time of the antitoxin in plasma produced. The horizontal dimension represents time, in days, since the commencement of immunization. The broken vertical lines represent amounts of toxin injected as represented by the coordinates at the right of the page; the solid curved line, the fluctuations in the titer (units); and the broken curved line the fluctuations in the inverse flocculation time (inverse hours) of the plasma as represented by the outside and inside values, respectively, at the left of the page.

0.01 cc. intervals. One-half cc. of a fresh, rapidly flocculating preparation of toxin, containing not less than 8 to 10 Lf units per cc., is added and the tubes gently shaken. The tubes are permitted to stand at room temperature and are centrifugated at intervals of not longer than 20 minutes. The tube in which a coherent sediment is first produced upon centrifugation is taken as the indicating time and Lf value tube. The sediments are readily visible against a dark back-ground when the tubes are shaken slightly during strong illumination from below.

The flocculation time should rise slowly and steadily from a value of about 1.0 to 1.5 hours at the end of the first month of immunization

to a final value of from 1.5-2.5 hours. A rapid decrease in flocculation time indicates that an insufficient amount of antigen is being given, and a rapid increase suggests that the animal is being undesirably "forced."

The Comparative Avidity and Therapeutic Usefulness of the Antitoxins Produced.—The titer of a serum, expressed in Ehrlich L_0 units, represents its total combining capacity and defines the quantity of toxin which can be neutralized by the serum when the mixture is allowed to reach combination equilibrium, either in vitro or in a subcutaneous wheal, preliminary to its diffusion into the tissues. In an actual diphtheritic infection, the toxin is already diffusing into the tissues at the time the antitoxin is administered. The injected antitoxin must compete for the toxin against fixation and differential diffusion processes that occur at a fairly rapid rate, and it is a question whether a static value like the titer, containing no reference to the speed with which the serum can neutralize toxin, really indicates its therapeutic usefulness. The avidity of a serum represents its total potential energy of combination, determines the speed with which it can neutralize toxin, and defines the amount of toxin which can be neutralized by the serum when opportunity for combination extends only over a specified, brief interval.

Avidity is defined as the product of titer and unit effectiveness, and the unit effectiveness of a freshly drawn antitoxin plasma may be gauged, roughly, by the shortness of its flocculation time with a standard toxin.

The time (t) at which flocculation may first be observed in a neutral toxin-antitoxin mixture may be assumed to be inversely proportional to a function of the combining energy or avidity: $(n/k') (K + a)$ of the mixture, where n is the number of units of toxin-antitoxin in the mixture, K and a are the combining avidities of the toxin and antitoxin respectively, per unit, and k' is a reference coefficient, i. e.,

$$C/t = e^{(n/k') (K + a)} \dots\dots\dots (1)$$

The constants C and K may be eliminated by the following subtraction:

$$\begin{array}{l} \log_e(C/t) = (n/k') (K + a) \\ \log_e(C/t') = (n/k') (K + a') \\ \hline \log_e(t'/t) = (n/k') (a - a') \dots\dots\dots (2) \end{array}$$

Let a' be the avidity of a one-minute serum ($t' = .017$ hrs.) and equal to 1, and let k' equal 0.25, a value which assumes zero avidity for a

flocculation time of 1,000 to 10,000 hours. The coefficient n has the value 2.86 for the toxin used, and:

$$a = .65 - .2 \log_{10} t \dots\dots\dots (3)$$

Equation (2) is, of course, imperfect and the values of a' and k' have been substituted in equation (3) somewhat empirically. The latter equation has been proposed only in order to define a relation between flocculation time and unit avidity which may be used as a provisional basis for comparisons of therapeutic effectiveness.

Values for the avidity, per unit, of the different plasmas collected during this study have been calculated from equation (3) and recorded in column six of table 1. The avidities of the plasmas, per cc. are recorded in column seven. An examination of this data suggests that the avidity, per unit, passes its maximum during the first month of immunization. This may be a consequence of the small size and wide spacing of the first few injections because a high unit avidity may be maintained by continuing the wide spacing of small injections throughout the course of immunization, as was done with horse 502. The unit avidity increased, in all the horses, whenever the close spacing of maximum injections was interrupted. It increased slightly during the interval between final injection and bleeding and also, to a very slight extent, between one large bleeding (without intermediate injection) and the next. These relations indicate that it may be difficult to produce a serum of maximum unit avidity and maximum titer simultaneously since the conditions under which unit avidity improves are exactly those under which maximum antitoxin production becomes interrupted.

It was not feasible to compare the therapeutic usefulness of all the plasmas for which the avidity is recorded. Therefore, a preparation from horse 502, having the highest unit avidity of 0.79, and preparations from horses 395 and 500, having the lowest unit avidities of 0.51 and 0.53, were chosen as extremes. They do not differ greatly and it is probable that the concentrated serums commercially available, representing as they do pooled and heated preparations, vary even less. The combining time for these preparations (column 3, table 2) was determined as the time required to elapse before a mixture of three units of the antitoxin with three L_0 units of the reference toxin could be injected into young rabbits without causing death. All injections were made intravenously with precaution against loss into the adjacent tissue and the quantities, spacings and times recorded are 80 to 90% accurate. The avidity per unit was calculated from the combining time

TABLE 1
THE ANTITOXIN TITER AND AVIDITY OF THE BLOOD PLASMA OF DIFFERENT HORSES AT
DIFFERENT STAGES OF THE IMMUNIZATION PROCEDURE

Horse	Injection Interval, Days	Total Toxin Injected, Lf Units	Plasma at End of Injection Interval			
			Titer, A, Units	Flocculation Time, Hours	Avidity	
					a, per Unit	aA per Ce.
457	0- 26	2300	18	0.25	0.77	14
	33- 49	10350	70	0.35	0.74	50
	54- 70	20800	175	1.1	0.64	110
	75- 91	32000	450	2.3	0.58	260
	96-113	44000	600	(350)
497	0- 30	1800	20	0.8	0.67	12
	35- 50	5900	200	2.8	0.57	110
	55- 71	10800	290	2.0	0.59	170
	76- 92	33400	900	2.0	0.59	530
	97-113	42400	1000	(550)
498	0- 9	150	5	(4)
	15- 37	3100	220	1.2	0.64	140
	43- 58	9100	600	1.4	0.62	370
	61- 79	17700				
	80	—	840	3.1	0.55	460
	82	—	900	2.4	0.57	510
	87	(Bled)*	850	2.3	0.58	490
	89	(Bled)	650	(390)
499	0- 9	150	22	0.25	0.77	17
	18- 30	1100	50	0.3	0.75	40
	36- 51	4700	90	0.6	0.69	60
	57- 72	16000	250	2.0	0.59	150
	78- 94	28800	600	(350)
500	0- 16	450	18	0.25	0.77	14
	22- 37	2300	140	3.5	0.54	80
	43- 58	9100	480	2.2	0.58	280
	64- 79	17700				
	80	—	525	6.3	0.49	230
	82	—	550	5.3	0.51	280
	85	(Bled)	600	5.5	0.50	300
	87	(Bled)	550	(280)
501	0- 18	700	20	0.4	0.72	15
	22- 38	2400	165	1.6	0.61	100
	44- 60	7100	430	1.8	0.60	260
	65- 80	14000				
	81	—	625	3.0	0.56	350
	85	(Bled)	840	2.8	0.57	490
	87	(Bled)	625	2.5	0.57	360
502	0- 25	490	90	0.15	0.83	70
	30- 47	2950	295	0.25	0.77	230
	53- 63	5100				
	73	(Bled)	340	0.20	0.79	270
503	0- 11	270	18	0.25	0.77	14
	16- 31	2600	260	1.2	0.64	170
	36- 54	7200	515	0.5	0.71	360
	59- 75	15800				
	80	(Bled)	1150	1.5	0.62	710
	82	(Bled)	850	1.7	0.60	510
	84	(Bled)	850	1.6	0.61	520
390	140-147	14500				
	152	(Bled)	750	2.9	0.56	420
	154	(Bled)	700	2.5	0.57	400
	156-168	24000				
	173	(Bled)	950	2.5	0.57	540
	175	(Bled)	900	2.0	0.59	530
	177	(Bled)	800	2.0	0.59	470
	180-189	16000				
	194	(Bled)	850	2.4	0.57	480
	196	(Bled)	825	2.4	0.57	470
	198	(Bled)	700	2.2	0.58	410
	201-210	22000				
	215	(Bled)	950	2.2	0.58	550
	217	(Bled)	800	2.0	0.59	470
	219	(Bled)	750	1.8	0.60	450

* Approximately one-third of the total blood volume of the horse was drawn at each bleeding recorded.

according to equation (3). The L_e unit is used in the table only for the sake of brevity and is not proposed as a new term. It represents the quotient of the L_o unit by the unit avidity a , i. e.:

$$L_e = L_o/a \dots\dots\dots (4)$$

The L_e or avidity unit was further determined, experimentally, as the quantity of antitoxin, in cc., required to neutralize one L_o unit of the same, fresh reference toxin when the two are injected into the blood stream of a rabbit immediately after mixture. Column six records the quantity of antitoxin required to neutralize one L_o unit of the toxin when the former is injected (intravenously) five minutes after the latter. It may be noted that when this quantity is expressed in L_o units, the three serums are of divergent unit protective power whereas when the quantity is expressed in L_e units, the protective power per unit

TABLE 2
THE RELATION OF AVIDITY TO THERAPEUTIC EFFECTIVENESS

Plasma from Horse	Ehrlich (L_o) Unit, Cc.	Com- bining Time, Hours	Avidity (L_e) Unit		Antitoxin Required to Save Rabbit 5 Min. after Intravenous Injection of 1 L_o Unit of Toxin		
			Calcu- lated, Cc.	As Ob- served, Cc.	Cc.	L_o Units	L_e Units
502	0.0028	0.2	0.0035	0.0036	0.66	240	190
500	0.0018	4.0	0.0034	0.0031	0.66	370	190
395	0.0038	5.0	0.0075	0.0067	1.54	410	210

is equal for any of the three serums. This finding may suggest that a preparation of antitoxin which is to be used for therapeutic purposes should be labelled not only with its content of Ehrlich L_o units but also with some avidity index, such as the combining time.

SUMMARY

The preparation of diphtheria antitoxin serums of high titer requires that the quantities of toxin injected during the course of immunization be large enough to maintain maximum production. An interruption of maximum production may be detected at once by a rapid decrease from the normal in the flocculation time of a trial bleeding. The flocculation time of the plasma ordinarily passes through a minimum value of about six minutes during the first month of immunization and then rises gradually to a value of about two hours at the end of the third month, although it may be maintained at the minimum value by restricting the amounts of toxin injected to quantities which just constitute an antigenic stimulus, or it may reach values upwards of four hours when the animal is being undesirably forced.

The avidity of a fresh antitoxin plasma, per unit of titer, is a function of the shortness of its time of flocculation with a given toxin. It has been evaluated for the plasmas drawn during this study and has varied from a maximum of 0.8 to a minimum of 0.5. Correspondingly, it has been found necessary to employ approximately 1.25 L_o units of the former preparation and 2.0 L_o units of the latter preparation to neutralize the toxicity of one L_o unit of toxin when the two were injected intravenously into rabbits immediately after mixture. When the times of injection of the toxin and antitoxin were separated by five minutes, approximately $1.25 \times 200 L_o$ units of the first preparation and $2.0 \times 200 L_o$ units of the latter were required.

THE DEGENERATION OF COMPLEMENT ON STORAGE AT SEVERAL TEMPERATURES

JOHN F. NORTON, BLAKE BARFIELD AND I. S. FALK

From the Department of Hygiene and Bacteriology of the University of Chicago

Pooled complement has proved to be more reliable in the Wassermann reaction than that obtained from a single animal. In small public health and diagnostic laboratories daily tests are often few in number and it would therefore simplify the procedure if this complement could be stored for use instead of being collected daily. The following experiments were made in an attempt to correlate the somewhat discordant results reported in the literature. Serums were obtained from guinea-pigs, rabbits, swine, horses and sheep.

The complementary activity of serum kept at room temperature or in the 37 C. incubator is known to decrease rapidly. Noguchi and Bronfenbrenner,¹ using guinea-pig serum, obtained a reduction in activity to two-fifths of the original after 6 hours storage at 37 C. and to one-half after 24 hours at 10 C. Brooks² records rapid deterioration at high temperature and slower reduction in activity at low temperatures. He also obtained evidence of regeneration of hemolytic power at 37 C. The effect of high temperature storage is so well recognized that it needs no further substantiation. The question of regeneration of activity, however, is an open one, in spite of Gramenitski's work.³

The effect of low temperature on complementary activity has been studied by a large number of investigators. Morgenroth⁴ stated that if complement is stored at —10 to —15 C., it retained its activity for a "long time." Penecke⁵ found that at these temperatures activity was maintained for four or five weeks. Weston,⁶ using —15 C., obtained no deterioration for three months, and one of three specimens retained its activity for 5 months. Bigger⁷ obtained similar results. Moledesky⁸ stored serum in vacuum bottles filled with ice and salt and found that it kept indefinitely. Kolmer, Matsumain and Krist⁹ found that complement stored for 3 days at room temperature deteriorated as much as that stored at 4 C. for two weeks and that frozen serum retained its activity still longer. They used both diluted and undiluted serum. Ruediger¹⁰ showed that complement stored at ordinary refrigerator temperatures lost its activity in less than two weeks. If frozen, the complement was worthless in three weeks. At very low temperatures it could be kept eight to ten weeks, deterioration beginning after about one month.

Received for publication, April 1, 1927.

¹ J. Exper. Med., 1911, 13, p. 78.

² J. M. Res., 1920, 41, p. 399.

³ Biochem. Ztschr., 1912, 38, p. 501.

⁴ Studies in Immunity, 1910, p. 329.

⁵ Ztschr. f. Immunitätsforsch. u. exper. Therap., 1913, 18, p. 112.

⁶ J. M. Res., 1915, 32, p. 391.

⁷ J. Path. & Bacteriol., 1919, 22, p. 323.

⁸ J. Am. M. A., 1918, 71, p. 968.

⁹ J. Syphilis, 1919, 3, p. 513.

¹⁰ J. Am. M. A., 1922, 79, p. 551.

Experimental Work.—In these experiments we have attempted to obtain definite figures on the rate of decrease of hemolytic activity of complement stored at 37 C, 4 to 6 C, and —10 C. Both diluted and undiluted serums were used. The serums were obtained aseptically. Pooled serums from guinea-pigs, rabbits and swine used but the specimens of horse serum were from single animals. Sheep serum would not complement the system used. This will be discussed later. Antisheep-cell hemolysin was used as antibody and carefully washed sheep-cells as antigen. All dilutions were made with 0.85% sodium chloride solution. Kolmer's¹¹ technic was followed and all reagents were tested in a Wassermann system.

TABLE 1
HEMOLYTIC POWER OF COMPLEMENT ON STORAGE AT 37 C.

Source	Dilution	Original Titer*	Titer After Storage (Days)								
			1	2	3	4	5	6	7	14	21-42
Guinea-pig...	Undiluted...	0.015	0.03	0.08	0.09	0.10	0.15	0.30	0.30	4.0	0
	Undiluted...	0.020	0.06	0.08	0.09	0.15	0.30	0.40	0.60	0	0
	1:20.....	0.30	0.40	0.50	1.0	3.0	4.0	5.0	0	0	0
	1:20.....	0.40	0.45	0.50	1.0	2.0	6.0	0	0	0	0
Rabbit.....	Undiluted...	0.04	0.05	0.09	0.20	0.30	0.40	0.60	0.75	5.0	0
	Undiluted...	0.025	0.04	0.05	0.07	0.09	0.10	0.30	0.50	1.0	0
	1:10.....	0.25	0.35	0.50	0.90	1.0	2.0	4.0	6.0	0	0
	1:10.....	0.40	0.60	0.90	2.0	4.0	5.0	7.0	0	0	0
Swine.....	Undiluted...	0.030	0.035	0.04	0.08	0.10	0.4	0.5	0.6	1.2	0
	Undiluted...	0.035	0.040	0.05	0.08	0.25	0.5	0.6	0.8	2.0	0
	1:10.....	0.30	0.40	0.50	0.80	1.0	2.0	5.0	0	0	0
	1:10.....	0.35	0.40	0.60	0.80	3.0	6.0	0	0	0	0
Horse.....	Undiluted...	0.06	0.09	0.15	0.30	0.8	2.0	4.0	6.0	0	0
	Undiluted...	0.07	0.10	0.20	0.35	0.7	1.0	3.0	6.0	0	0
	1:5.....	0.30	0.50	0.80	1.0	1.5	3.0	5.0	0	0	0
	1:5.....	0.35	0.55	0.75	0.9	2.0	3.0	5.0	9.0	0	0

* Complementary unit expressed in cc. of serum as stored, and 0 equals titer too low to determine, in all tables.

Tables 1, 2 and 3 show the changes in hemolytic activity of complement stored for 42 days at three temperatures on two specimens of blood from each of four species of animals. Actually, seven tests were made on both diluted and undiluted guinea-pig serum, and five tests on rabbit serum. The results were so concordant that the complete series is not here recorded.

Rough graphs indicated that the rate of deterioration followed a logarithmic course. This can be represented by the equation $K = \frac{2.3}{t} (\log a - \log b)$ in which t represents the time interval between two consecutive titrations, a — the number of units at the beginning, and b — the number of units at the end of the time interval, and K is a constant.

¹¹ Kolmer and Boerner: Lab. Diag. Methods, 1925.

TABLE 2
HEMOLYTIC POWER OF COMPLEMENT ON STORAGE AT 4 TO 6 C.

Source and Dilution	Original Titer	Titer After Storage (Days)											
		1	2	3	4	5	6	7	14	21	28	35	42
Guinea-pig:													
Undiluted..	0.030	0.030	0.030	0.040	0.045	0.060	0.065	0.07	0.270	0.3	0.6	1.0	2.0
Undiluted..	0.030	0.035	0.035	0.035	0.050	0.080	0.080	0.09	0.270	0.6	1.0	2.0	2.0
1:20.....	0.30	0.30	0.30	0.35	0.45	0.8	1.0	1.5	3.4	4.0	0	0	0
1:20.....	0.40	0.40	0.40	0.50	1.0	1.5	2.0	3.6	6.0	7.0	0	0	0
Rabbit:													
Undiluted..	0.04	0.04	0.05	0.06	0.07	0.09	0.10	0.15	0.40	0.85	2.5	0	0
Undiluted..	0.025	0.025	0.030	0.035	0.045	0.055	0.06	0.07	0.25	0.50	1.0	2.0	0
1:10.....	0.25	0.25	0.35	0.45	0.70	0.80	1.5	2.0	6.0	0	0	0	0
1:10.....	0.40	0.50	0.70	0.80	1.0	1.5	2.5	3.5	7.0	0	0	0	0
Swine:													
Undiluted..	0.030	0.030	0.030	0.05	0.06	0.07	0.08	0.09	0.20	0.60	1.0	0	0
Undiluted..	0.035	0.035	0.040	0.050	0.07	0.08	0.09	0.10	0.25	0.80	3.5	0	0
1:10.....	0.30	0.45	0.45	0.55	0.55	0.60	0.70	2.0	5.0	0	0	0	0
1:10.....	0.35	0.35	0.50	0.55	0.60	0.60	0.80	3.0	0	0	0	0	0
Horse:													
Undiluted..	0.06	0.06	0.07	0.075	0.08	0.09	0.10	0.18	0.50	1.2	0	0	0
Undiluted..	0.07	0.07	0.07	0.085	0.10	0.15	0.20	0.25	0.65	2.0	4.0	0	0
1:5.....	0.30	0.30	0.50	0.80	0.90	1.0	2.0	4.0	6.0	0	0	0	0
1:5.....	0.35	0.35	0.40	0.50	0.80	1.0	2.0	4.0	8.0	0	0	0	0

TABLE 3
HEMOLYTIC POWER OF COMPLEMENT ON STORAGE AT -10 C.

Source and Dilution	Original Titer	Titer After Storage (Days)											
		1	2	3	4	5	6	7	14	21	28	35	42
Guinea-pig:													
Undiluted..	0.020	0.020	0.020	0.025	0.030	0.03	0.04	0.04	0.10	0.20	0.60	0.80	1.0
Undiluted..	0.035	0.035	0.035	0.040	0.080	0.08	0.09	0.10	0.18	0.50	0.90	2.0	2.0
1:20.....	0.35	0.35	0.35	0.35	0.50	0.50	0.90	0.90	1.8	3.0	5.0	0	0
1:20.....	0.30	0.30	0.35	0.40	0.60	0.70	0.70	1.0	3.0	4.0	8.0	0	0
Rabbit:													
Undiluted..	0.040	0.040	0.040	0.045	0.050	0.060	0.070	0.08	0.22	0.45	0.9	1.5	0
Undiluted..	0.025	0.025	0.025	0.030	0.030	0.035	0.040	0.05	0.15	0.30	0.6	0.9	0
1:10.....	0.25	0.25	0.30	0.35	0.50	0.60	0.65	0.70	3.0	6.0	0	0	0
1:10.....	0.40	0.45	0.45	0.50	0.80	0.90	0.95	1.2	5.0	0	0	0	0
Swine:													
Undiluted..	0.030	0.030	0.035	0.035	0.04	0.05	0.06	0.06	0.18	0.5	0.6	2.0	0
Undiluted..	0.035	0.035	0.040	0.045	0.06	0.065	0.07	0.08	0.20	0.5	0.9	3.0	0
1:10.....	0.30	0.30	0.35	0.35	0.40	0.50	0.70	0.90	2.0	4.5	0	0	0
1:10.....	0.35	0.35	0.35	0.40	0.50	0.70	0.80	1.0	3.0	5.0	0	0	0
Horse:													
Undiluted..	0.06	0.06	0.07	0.075	0.08	0.09	0.10	0.12	0.30	0.9	2.0	4.0	0
Undiluted..	0.07	0.07	0.07	0.080	0.09	0.10	0.10	0.15	0.40	1.0	3.0	6.0	0
1:5.....	0.30	0.30	0.35	0.40	0.50	0.55	0.70	0.9	2.0	0	0	0	0
1:5.....	0.35	0.35	0.40	0.45	0.60	0.70	0.90	1.0	3.0	0	0	0	0

The value for K for each temperature and for each serum, both undiluted and diluted are given in table 4. All of our experiments were used in these calculations.

The results recorded in table 4 show that K is reasonably constant at a given temperature and is independent of the source of the serum. Deterioration of diluted serum is somewhat more rapid than that of the undiluted serum.

Sheep Serum.—It was noted that sheep serum did not contain complement for the hemolytic system used. Either the serum was anticomplementary or it actually lacked the complementary factor or it contained substances which interfered with the action of the hemolysin or of the antigen used in the system.

TABLE 4
CONSTANTS CORRESPONDING TO THE VELOCITY OF A MONOMOLECULAR REACTION

Serums	K		
	—10 C.	4 to 6 C.	37 C.
Undiluted:			
Guinea-pig.....	.00671	.00709	.0177
Rabbit.....	.00575	.00767	.0110
Swine.....	.00480	.00547	.0177
Horse.....	.00480	.00751	.0272
Average.....	.00552	.00694	.0184
Diluted:			
Guinea-pig.....	.00982	.0125	.0182
Rabbit.....	.00751	.0141	.0187
Swine.....	.00763	.0149	.0195
Horse.....	.00830	.0165	.0192
Average.....	.00832	.0145	.0189

The immune serum contained agglutinins for sheep cells to a titer of 1:10,000. Absorption experiments with the immune serum and washed sheep cells left a supernatant liquid which contained no hemolysin when tested with guinea-pig serum or sheep serum. The sensitized sheep cells were hemolyzed on the addition of guinea-pig serum but not with sheep serum. Serum proteins were precipitated from normal sheep serum by half saturation with ammonium sulphate (globulins) and then by complete saturation (serum albumin). The precipitates were dialyzed and dried at 37 C. under reduced pressure. These were then added to the immune serum and the heavy precipitate which formed was removed by centrifugation. The supernatant liquid was tested for the presence of hemolysin. Hemolysis occurred when guinea-pig serum was used as complement but not when sheep serum

was used. The sheep serum proteins apparently had not removed hemolysin from the immune serum.

Possible anticomplementary action of the normal sheep serum was tested as follows. A fresh portion of antish sheep cell serum was obtained from an immunized rabbit. Part of this was inactivated by heating at 56 C. for 30 minutes and part was left unheated. The hemolytic unit of the inactivated serum, using guinea-pig complement, was found to be 0.5 cc. of a 1:5,000 dilution. The unit of the unheated serum was 0.5 of a 1:6,000 dilution. The difference was due to the presence of rabbit complement in the unheated immune serum, or to the partial destruction of lytic potency by the heating. The unit of the unheated immune serum, using no complement other than that normally present in this serum, was 0.1 cc. of a 1:100 dilution. When normal sheep serum was added to this system the unit remained unchanged. The sheep serum therefore contained no anticomplementary substances and did not add any complement to the system.

These experiments indicate that the sheep serum in our possession contained no substances which interfered with hemolysis. Neither did it contain the complementary factor.

CONCLUSIONS

We have found no evidence of the regeneration of complement during storage over a period of 42 days at temperatures of 37, 4 to 6, and —10 C. Complement stored at 37 C. deteriorated rapidly. The activity of both undiluted and diluted serum disappeared by the end of the third day of storage at this temperature. Both undiluted and diluted serums retained their original complementary activity for 48 hours at 4 to 6 C. At the end of 7 days the unit was found to be two to three times as great as that of the fresh serum. Frozen serum kept at —10 C. retained its complementary power for two days, after which deterioration proceeded slowly. The undiluted serum could be used for about two weeks, the diluted serum for not more than five to seven days.

Guinea-pig serum possessed the greatest complementary potency. Swine serum ranked second. Horse and rabbit serums could be used but were less satisfactory. Sheep serum failed to complement the hemolytic system studied, due to the absence of the complementary factor rather than to the presence of interfering substances.

The rate of deterioration of complementary activity could be expressed by use of a monomolecular equation, although it does not necessarily follow that the reaction is monomolecular.

The constants obtained with this equation were approximately the same for the four serums stored at any one temperature, showing that the rate of loss of complementary activity was nearly identical for guinea-pig, swine, horse and rabbit serums. This may be considered as evidence in favor of the view that the complementary factor is the same, regardless of its source.

TUBERCULOSIS IN GUINEA-PIGS AFTER TREATMENT WITH TUBERCLE BACILLI MADE NON- ACIDFAST WITH OLEIC ACID

F. A. MCJUNKIN

From the Department of Pathology, Washington University School of Medicine, St. Louis, Mo.

In experiments previously reported by the author¹ it was determined that dehydrated bacilli suspended in oleic acid or in olive oil lose their acidfastness when placed in the incubator. Killed bacilli did not undergo the change. Loss of acidfastness took place most rapidly at 37 C. with an abrupt cessation at lower temperatures, when the metabolic activities of the bacilli were greatly reduced. At temperatures above 37 C. which approached the thermal deathpoint of the cultures the bacilli were affected but little. Under the conditions of these experiments, living cultures were regarded as necessary for the removal of acidfastness. Since the publication of these experiments, it has been found that the discharge of acidfastness can be accomplished without dehydration and suspension of the dehydrated bacilli in the oily medium. The purpose of this paper is to describe a procedure for rendering cultures in aqueous-alcohol suspensions nonacidfast by means of oleic acid, and to describe the tuberculosis of guinea-pigs treated with the liquid containing all substances removed from the bacilli during the removal of the acidfastness as well as the suspended nonacidfast bacilli. Treatment with the non-acidfast bacilli alone appears not to be effective in increasing the resistance of guinea-pigs to tuberculosis when subsequently inoculated with living bacilli.

*Preparation of Non-Acidfast Tubercle Bacilli.**—The method of removing the acidfastness of the bacilli avoids the use of the large quantity of oleic acid which was used in the earlier method, but it seems probable that the individual bacilli are placed in about the same environment so far as oleic acid, alcohol and water are concerned as were the dehydrated bacilli which were placed in the large volumes of oleic acid in the older method of suspension. In the older method¹ the bacilli were dehydrated with alcohol and at once covered with oleic

Received for publication, April 6, 1927.

¹ Am. Rev. Tuberc., 1923, 8, p. 393; 1924, 9, p. 464; J. Infect. Dis., 1926, 38, pp. 520, 524.

* A human type of tubercle bacilli (H 60 of the Saranac Laboratory) has been used in all experiments.

acid having a small amount of water in suspension. Under these conditions the bacilli and masses of bacilli suspended in the oleic acid were probably enclosed in an envelope of alcohol and water in which oleic acid was dissolved in amounts dependent upon the percentage of alcohol in the envelope. By the method now to be described, the cultures are covered with alcohol of a definite strength in which oleic acid is dissolved.

Method: The bouillon is decanted completely from a 100 cc. Erlenmeyer flask culture and 10 cc. of 2% oleic acid in 80% ethyl alcohol are added. This in turn is immediately decanted. The growth is again covered with 10 cc. of the alcoholic solution of oleic acid and placed at 37 C. If the growth of bacilli has not formed an even and heavy layer over the entire surface of the glycerol broth the pellicle is transferred from other flasks to make up the deficiency. After three days in the incubator, the culture is examined for loss of acid-fastness by making a heavy smear near the end of a slide, fixing it in the flame, pouring xylol over it to remove completely the oleic acid and then staining by the Ziehl-Neelsen method in the usual way. The bacilli should be more than 99% non-acidfast. After the addition of phenolphthalein indicator 15 to 17 drops of normal sodium hydrate are mixed with the suspension to neutralize the oleic acid. The reaction has usually been carried slightly to the alkaline side. The neutralized suspension is kept at 37 C. over night and then heated for 15 minutes in an Arnold sterilizer. Seventy cc. of distilled water are added and the heating continued for an additional 30 minutes. The sterilized culture is kept at 37 C. for about 48 hours and frequently agitated to mix the undissolved culture with the liquid. During this incubation many of the non-acidfast bacilli dissolve. At the end of two days the clearer supernatant portion is removed to a sterile flask. In smears made by evaporating drops of the liquid there are occasional acidfast rods and a variable number of blue-staining more or less amorphous rods. If the number of acidfast bacilli is too large the procedure should be repeated with new cultures. Non-acidfast bacilli suspended in the liquid are not objectionable as they are readily removed from the site of injection.

As in the earlier method, heat-killed bacilli are not acted upon. Cultures killed by heating in an autoclave for 10 minutes at 15 pounds pressure remain acidfast when treated by the technic described in the preceding paragraph. Correct concentration of the alcohol is necessary. If its strength be reduced to 60% cloudiness appears in the liquid and the loss of acid-fastness is incomplete. In a solution of oleic acid in 90% alcohol practically all of the bacilli remain acidfast. In all instances the dilutions of alcohol have been made from pure absolute ethyl alcohol.

Treatment of Guinea-Pigs with the Non-Acidfast Bacilli and Subsequent Inoculation with Living Cultures.—The injections have been made intraperitoneally. The concentration of sodium oleate is about a quarter percent and its irritant action may be made less by further

dilution with distilled water. After one month of injections at four or five day intervals the intracutaneous tuberculin test becomes strongly positive. The suspensions contain the entire substance of the bacilli.

A preliminary experiment was made with 10 guinea-pigs varying in weight from 685 to 850 grams. On July 8, 12 and 15, 1926, six of these were given intraperitoneal doses of 2.5 cc. of the suspension of bacilli. On August 14, these six together with four normal control guinea-pigs were inoculated intraperitoneally with a suspension of living bacilli showing about one bacillus per oil-immersion field. On October 4, the ten were killed. All were tuberculous. The gross and histologic differences between the controls and the animals receiving preliminary treatment with the non-acidfast bacilli were about the same as those recorded in table 1. The smaller size of the control guinea-pigs diminished the significance of these differences.

Ten of the sixteen guinea-pigs in the second group (table 1) were given intraperitoneally a total of seven 1 cc. injections of the non-acidfast bacilli on Nov. 23, 27, and 30, and on Dec. 4, 16, 20, and 24, 1926. Every guinea-pig increased in weight during the period of treatment with non-acidfast bacilli. Jan. 5, 1927, the ten together with six normal control guinea-pigs were inoculated intraperitoneally with 1 cc. of a suspension of living bacilli. The bacilli were first suspended by rubbing a small bit of culture in a mortar with a small quantity of 10% gelatin. After diluting the gelatin with physiologic salt solution the heavier masses were thrown down by centrifugation. In preparations made by evaporating drops of the supernatant liquid there were about 5 bacilli per oil immersion field. Before injecting it, this suspension was diluted one hundred times.

Results.—Non-tuberculous guinea-pigs have little natural resistance to experimental infection with virulent tubercle bacilli. This animal has frequently been used for experiments attempting artificial immunization without the use of living bacilli. Many of the procedures employed have been long and complicated, and obviously no analysis of the literature is possible in this short paper. It may be stated, however, that reports of artificial immunity in non tuberculous guinea-pigs lack satisfactory and conclusive confirmation. In view of these results, it is desirable to evaluate conservatively the differences between the control animals and those treated with the non-acidfast bacilli. The six controls (11 to 16 inclusive) and the guinea-pigs treated with the non-acidfast bacilli (1 to 10 inclusive) were selected so that there were heavy as well as light ones. The average weight of the former at the time of

TABLE 1
EFFECT OF TREATMENT WITH NONACIDFAST TUBERCLE BACILLI ON RESPONSE OF GUINEA-PIGS
TO INOCULATION WITH VIRULENT BACILLI

Guinea- pig	Weight at Time of Inoculation, at Gm. Jan. 5, of Ne-		Signs of Tuberculosis in				Remarks
	Jan. 5,	Time of Ne- cropsy, Gm. Jan. 31	Spleen	Liver	Lung	Omentum	
1	880	913 Jan. 31	Normal size 0*	0	0	Normal thick- ness; one very small clump of lymphocytes and mononu- clear cells in one section	
2	800	800 Jan. 29	Normal size 0	0	0	Normal thick- ness; small tubercles	
3	900	968 Jan. 31	Normal size 0	0	0	Normal thick- ness; small foci of round cells on surface	
4	865	850 Jan. 31	Moderately enlarged	No tubercles in gross	One tubercle in 2 cm. section	Normal thick- ness; 0	Fibrosis about spleen tubercles
5	975	980 Jan. 31	Moderately enlarged	0	0	Normal thick- ness; scatter- ed small tu- bercles on surface	Fibrosis in periphery of spleen
6	1225	1260 Jan. 31	Normal size 0	0	0	Normal thick- ness; very small foci of round cells on surface	
7	555	565 Jan. 29	Moderately enlarged	Two tubercles in 2 cm. section	One tubercle in 2 cm. section	Normal thick- ness; few small tu- bercles	Fibrosis of spleen tu- bercles
8	675	680 Jan. 29	Normal size 0	0	?	Normal thick- ness; few tubercles	Foci of or- ganizing pneumonia in lung
9	660	775 Jan. 29	Normal size; one tubercle in 2 cm. section	One tubercle ?	0	Normal thick- ness; scatter- ed tubercles	Fibrosis
10	525	530 Jan. 29	Normal size	One in 2 cm. section	0	Thickened; ex- tensive tu- berculosis	Fibrosis about spleen tubercles
CONTROLS NOT TREATED WITH NONACIDFAST BACILLI							
11	998	998 Jan. 31	Enlarged	One tubercle per section	Two tubercles per section	Omentum thick; solid mass of tu- bercles	No fibrosis in spleen
12	900	855 Jan. 31	Enlarged	One tubercle per section	Two tubercles per section	Omentum enormous; solid mass of tubercles	No fibrosis in spleen
13	670	625 Jan. 31	Enlarged numerous tubercles	Six tubercles per field	One small tubercle in section	Omentum great hard mass	Spleen not examined microscop- ically
14	720	775 Jan. 31	Enlarged	Three tuber- cles in section	Twelve small tubercles in section	Omentum en- larged; ex- tensive tu- berculosis	No fibrosis in spleen
15	830	830 Jan. 26	Moderately enlarged; numerous tubercles	Four tuber- cles in section	Ten tuber- cles in section	Omentum enormous; extensive tu- berculosis	Died from the tuberculosis on Jan. 26; no fibrosis in spleen
16	910	885 Jan. 31	Enormous spleen	One per field	Four tuber- cles per field	Omentum enormous; extensive tu- berculosis	No fibrosis in spleen

* 0 indicates absence of well defined foci of epithelioid cells, foreign-body giant cells, and necrosis. The microscopic lesion in the spleen varied in character but in the average control was more extensive than in the average treated animal. Also in several of the treated animals there was a tendency to formation of reticular or fibrous tissue.

inoculation with living bacilli was 838 gm. while that of those receiving preliminary treatment with non-acidfast bacilli was 806 gm. The three guinea-pigs weighing least (7, 9 and 10) were in the group receiving non-acidfast bacilli. The spleens of these three were about normal in size, and in each there was on microscopic examination a zone of fibrosis at the periphery of the tubercles. In only one (10) of the three was the omentum materially thickened. In the remaining seven larger guinea-pigs receiving preliminary injections of non-acidfast bacilli the spleens were of normal size and the omentums showed no distinct thickening on naked-eye inspection. In those of the seven in which splenic tuberculosis was present microscopically the peripheral portions of the tubercles were fibrous and in several instances densely fibrous. In five (the block of spleen from the sixth one was accidentally lost) untreated control animals the fibrosis (reticulosis) was almost completely lacking. In only one instance have the large mononuclear cells of the epithelioid type lengthened so as to assume some of the characters of reticular cells. In four (1, 2, 6 and 8) of the groups receiving preliminary injections of non-acidfast bacilli active tuberculosis was not conclusively demonstrated by the histological examinations made. Smears stained for tubercle bacilli were not examined. The small collections of cells on the omental surfaces no doubt were foci of infection. With infection established the tuberculosis probably would have progressed during the next few weeks to such an extent that the difference in the lesions of treated and untreated animals would have been obscured. Young guinea-pigs are not suitable for the experiment.

Further investigation may develop a procedure for decreasing the percentage of objectionable sodium oleate and at the same time concentrating the quantity of non-acidfast bacilli in the liquid. The non-acidfast bacilli are absorbed rapidly and the local irritation depends on the concentration of the oleate. Presumably the injection of a greater quantity of non-acidfast bacilli without corresponding increase of the oleate would heighten the resistance to the infection. If heat-killed tubercle bacilli are injected into normal animals they remain in the tissues undissolved for a long time.² If large doses of the acidfast bacilli are injected a chronic local reaction slowly develops which is associated with loss of weight and health. It was found that olive oil retarded the absorption of the non-acidfast tubercle bacilli and that guinea-pigs receiving preliminary injections of mixtures of non-acidfast bacilli and olive oil were not more resistant than normal animals to tuberculosis.¹

² Prudden, T. M., and Hodenpyl, E.: N. Y. M. J., 1891, 53, p. 697.

CONCLUSIONS

Cultures of tubercle bacilli may be made non-acidfast by incubating them with 2% oleic acid in 80% ethyl alcohol, and by neutralization of the oleic acid with sodium hydrate and by dilution with distilled water there may be obtained moderately heavy suspensions of the non-acidfast bacilli in a concentration of about one-fourth % of sodium oleate.

The suspension of non-acidfast tubercle bacilli may be injected into large guinea-pigs without loss of weight and with no adverse effect on their general health, and guinea-pigs so treated become infected when subsequently inoculated with living bacilli, but the infection appears to progress less rapidly with the formation of fewer and smaller tubercles in the organs and with greater transformation of epithelioid cells into reticular tissue, than in untreated guinea-pigs. From the data now at hand it seems probable that the favorable effect of the treatment is seen best in large guinea-pigs about four weeks after inoculation.

A PHARMACO-BACTERIOLOGIC STUDY OF AFRICAN POISONED ARROWS

IVAN C. HALL AND RICHARD W. WHITEHEAD

From the Departments of Bacteriology and Public Health and of Physiology and Pharmacology, University of Colorado Medical School, Denver

It has long been known that arrow wounds are likely to become infected if they do not immediately prove fatal through trauma or through the action of specific poisons.

Ledantec in 1890 pointed out that tetanus frequently occurred after arrow wounds in the New Hebrides, and succeeded in reproducing tetanus in guinea-pigs by direct inoculation of the arrow poison. In 1892 he found that certain animals died with the symptoms of septic gangrene after inoculation with certain lots of arrow poison from the New Hebrides or cultures therefrom which resembled the *Vibrio septique* of Pasteur. He ascribed these germs to contamination of the arrows with soil from marshes.¹ In spite of this propitious beginning there is relatively little known as to the bacteriology of arrow poisons or arrow wounds. Parke² suspected tetanus in some of his men who died following arrow wounds, but as the result of a study of the plants used in the preparation of arrows by the pygmies of Central Africa, in collaboration with Holmes,³ the tetanic symptoms were finally ascribed to strychnine from a species of *Strychnos*, and when Lewin⁴ wrote his now classical dissertation upon arrow poisons, the possibility of their effects being due to, or complicated by, bacterial infections was practically ignored. There have been, however, a few contributions since Lewin's thesis which suggest the importance of this view point. Thus Mines⁵ said in discussing the Munchi arrow poison, "It is said that some tribes attempt to make their weapons still more deadly by placing the poisoned arrow heads in the flesh of putrefying corpses. They thus become carriers of bacterial infection. In this connection it is of interest to note that the extracts of the Munchi arrow poison acquired a putrefactive odour within a week, unless they were boiled, in which case they often remained unaltered for over a month." Parsons⁶ also recorded a similar observation in Northern Nigeria, decomposing animal secretions and carcasses serving as a sort of pincushion for infecting the arrows. Further, "where the tetanus bacillus abounds arrows are rendered poisonous by merely planting them head downwards in the soil." "No surgically clean arrow ever leaves the bow string, so that sepsis has to be guarded against in any case." This writer emphasized the treating of all arrow wounds as infected wounds and listed ten cases of men wounded with arrows, of whom five died in one-half to one and one-half hours, and five died in thirty hours to eight days, as follows: thirty hours,

Received for publication, April 11, 1927.

¹ Ann. de l'Inst. Pasteur, 1890, 4, p. 716; 1892, 6, p. 851.

² My Personal Expedition in Equatorial Africa, 1891.

³ Pharm. J., 1891, Vol. 2, p. 917.

⁴ Die Pfeilgifte: Histrionische und Experimentelle Untersuchungen, 1894.

⁵ J. Physiol., 1908, 37, p. 37.

⁶ Brit. M. J., 1909, 1, p. 212.

cellulitis and meningitis; thirty-six hours, cellulitis; five days, cellulitis; seven days, cellulitis (cured?), tetanus; and eight days, tetanus.

Anderson⁷ studied arrows and arrow wounds in Manbhum, India. "The following are the chief methods of poisoning the arrow head 1. By placing the tip of the head into rotten flesh or meat or into the flesh of a much decomposed human body. 2. By smearing the head with a mixture of nux vomica and sulphide of arsenic. 3. By smearing the tip and edges with snake venom or painting the whole head with the venom. 4. By placing the head into animal secretions in a state of decomposition or smearing the tip with tiger's blood, if obtainable. 5. Certain tribes simply dip the head into mud, whilst the arrows themselves are frequently laid on the ground and are almost certain to infect a wound produced by them with tetanus, a disease which is extremely common in tropical countries." Anderson also mentioned three types of result, first, sudden death due to heart failure or trauma, second, spasms due to strychnine or tetanus, third, infective complications such as sepsis, erysipelas, cellulitis, edema and gangrene after several hours or days.

Turning to Perrot and Vogt⁸ whose well illustrated and comprehensive treatise must be regarded as the best and most authoritative modern source of information upon the general subject of arrow poisons, we find the idea of bacterial infection clearly recognized in the following words, "Parfois même et c'est encore un fait bien curieux, les armes sont enduites de véritables cultures microbiennes naturelles (Vibron septique, bacille du tetanos)." This obviously refers to the work of Ledantec. Castellani and Chalmers,⁹ also, refer briefly to the problem of bacterial infection. "The Bushmen of the Kalahari Desert make a poison from the Larvae of *Diamphidia simplex* Paringuey which is thought to be really a toxin due to some microorganism growing in the decomposing larvac." Referring to the use of aconite in Asia, "the poison is applied as a paste to the arrow and is said to be mixed with septic blood to increase its effect." Castellani and Chalmers recommend tetanus antitoxin in the routine treatment of arrow wounds.

Yet in none of the available literature other than Ledantec's papers was there any reference to actual bacteriologic studies of arrow poisons or laboratory diagnosis of the infections caused by arrow wounds. Therefore, when an opportunity was presented through the courtesy of Dr. C. Ernest Cadle, Director of the Denver African Expedition of 1925, to study several arrows procured by him from the African bushmen, it was seized with avidity. The results of our study point to the existence of pathogenic bacteria upon arrows and in arrow wounds similar to those in gun shot wounds. That death through arrow wounds is not even yet a thing of the past is shown by the fact that one of Dr. Cadle's best friends was fatally shot by a poisoned arrow in Africa, and any knowledge concerning the etiology of arrow wounds is fully justified by the increased frequency with which members of the white race are now invading Africa for exploratory, predatory or other reasons.

⁷ Indian M. Gaz., 1911, 46, p. 10.

⁸ Poisons de Flèches et Poisons d'Épreuve, 1913.

⁹ Manual of Tropical Medicine, 1919.

Description of the Arrows.—There were six arrows, three from the Kung or Kalahari pygmy bushmen, one from the Ovachimba tribe of the Kaokoveldt, and two from the Heikum bushmen.

The Kung or Kalahari arrows are made of a light yellow bamboo reed, about 0.7 cm. in diameter and vary in total length from 54 to 63 cm., including the points, which are of ivory or bone and of which 8 to 11.5 cm. extend beyond the hollow reeds into which they were inserted and to which they were bound by means of fine sinews. The other ends of the reeds are notched to receive the bow string and bound also with sinews to prevent splitting. They are not feathered. One of these arrows is also bound in two places along the shaft. It was impossible to detect any extraneous (poisonous?) material upon these arrows by the naked eye.

The Ovachimba arrow consists of a notched hard wood stick 0.8 cm. in diameter, and 70 cm. in length, which is inserted into the hollow shaft of a flat rusty barbed iron point 2.5 cm. broad and 7.0 cm. long. The shaft near the barb was stained brown but there was no considerable amount of material sticking to either shaft or point that could be suspected of being poisonous. This arrow has the partially stripped shafts of brown feathers bound to the notched end with sinews. Being parallel to the shaft, they would tend to prevent rotation of the arrow.

The Heikum arrows are alike in construction but slightly different in size. The larger is a hard wood stick 60 cm. long and 0.75 cm. in diameter, tipped with a flat barbed iron point 2.5 cm. long and 2 cm. broad, with its shaft inserted into the split stick and the latter bound tightly to it with sinew. The point and binding are pasted heavily with a dark muddy like substance full of grit. The smaller Heikum arrow is a hard wood stick 55 cm. long and 0.6 cm. in diameter, tipped with a flat iron point 2 cm. long and 1.2 cm. wide whose shaft is inserted into the split stick and the latter bound tightly to it with sinew. There is no poison on the point but that portion of the shaft bound to the point is covered with a black, hard paste. Both arrows are feathered with six partially stripped brown feathers bound straight to the shafts with sinew. No rotation of the arrow would be imparted by them. All of the arrows are shown in figure 1.

Dr. Cadle has shown to the writers moving pictures of the Heikum bushmen engaged in the manufacture of their poison arrows for which purpose they use the juice of the succulent roots of certain plants boiled down to a thick paste. Figure 2 illustrates a bushman boiling the poison, as well as some of the roots from which it is made. According to Dr. Cadle, different tribes of bushmen use different poisons. Snake venoms are used only when the plant poisons are unavailable, being regarded by the bushmen as too slow in action. But when snake venoms are used, they are mixed with the juices of *Euphorbia*, *Bufane distichia*, or *Strychnos toxifera*. In the Etosha Pan region the Heikum bushmen use only *Euphorbia*, whether alone or with animal poisons could not be determined. The Kung bushmen, one hundred miles northeast of Grootfontein, use the above vegetable poisons, together with the venoms

of horned and puff adders plus the bodies of insect larvae. The mixture is boiled down in a hollow stone into which the poison maker frequently spits during the intervals in a weird chant. The boiling no doubt greatly impairs the efficacy of the snake venoms. After about ten minutes' boiling, the paste is carefully smeared onto the arrow with a bone instrument or grooved stone, care being taken that the poison never touches the fingers. This account essentially verifies the statements made by Farini,¹⁰ Lewin,¹ Stow,¹¹ Perrot and Vogt⁸ and Dornan.¹²

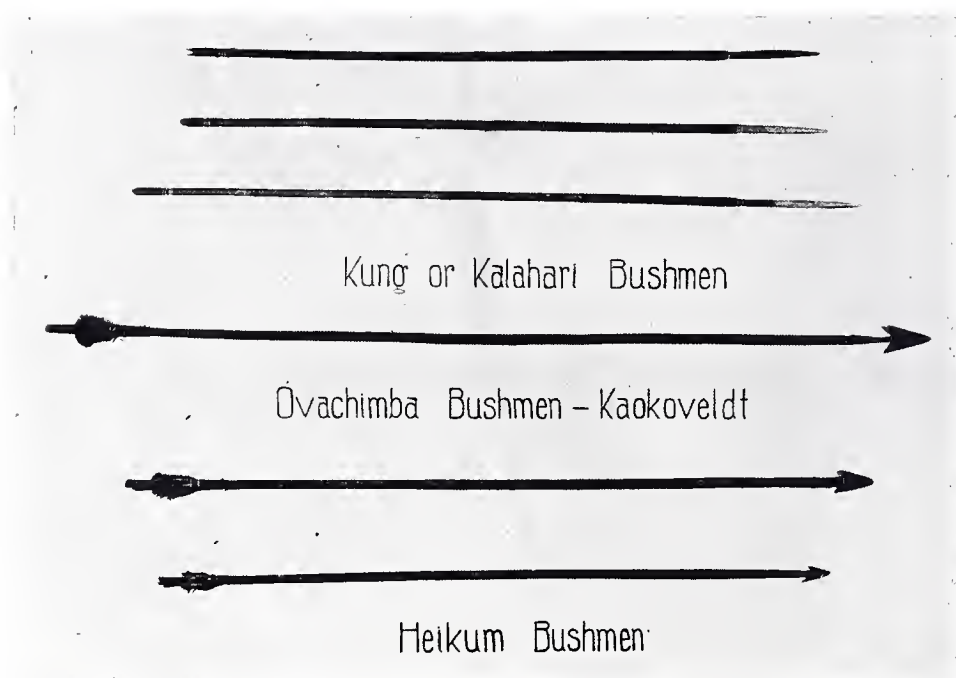


Fig. 1.—African poisoned arrows.

Lewin¹ recorded that the bushmen use the poison fangs and glands of snakes, notably the puff adder (*Echidna arietans*) and of the cobra (*Naje Haye*) which are mixed with the poisonous amaryllis (*Haemanthus toxicarius*, or *Amaryllis disticha*) as well as with several species of *Euphorbia*, and the leaves, rind, wood and fruit of *Acokanthera venenata*. Lewin found the poison from a museum arrow known to be eighty-eight years old, still active in small doses for rabbits, pigeons and frogs. He also investigated the toxicity of the larvae of *Diamphidia simplex* (Peringuey), which he found to be a mild toxalbumen. Both

¹⁰ Through the Kalahari Desert, 1886.

¹¹ The Native Races of South Africa, 1905.

¹² Pygmies and Bushmen of the Kalahari, 1925.

the larvae and the adult beetles are used by the Kalahari bushmen on their arrows. Perrot and Vogt's ⁸ list differs from that of Lewin mainly in nomenclatural changes. It includes the snakes, *Echidna arietans* Gray, and *Naja tripudians* Merr. (*Coluber-naja* L.), the beetle *Diamphidia locusta* Fairm., and the plants, *Bufane disticha* Herb. (= *Haemanthus toxicarius* Ait.= *Amaryllis disticha* L.), *Euphorbia cereiformis* L., *E. virosa* Willd., *E. heptagona* L., *E. arborescens* (?),



Fig. 2.—Heikum bushman preparing arrow poison from euphorbia roots.

and *Acokanthera venenata* (Thbg) Don. These lists indicate the complex nature of the arrow poison and suggest, as already noted, that its composition must vary with the different tribes and indeed even within the same tribe. Dornan ¹² gives the best recent account of the life of these people.

Dr. Cadle found, in tests made upon the field, that the effect of the arrows upon wild animals varied according to their size. Springboks (*Antidorcas euchore*) sometimes succumbed within two hours, while the eland (*Taurotragus oryx*) often survived for ten hours, and a

giraffe would go fifty hours. In all cases the animals suffered violent paroxysms of pain, and the site of the wound became tumefied. The natives cut out such areas before consuming the flesh.

These facts, to a bacteriologist, indicate infection rather than, or at least in addition to, direct poisoning.

Preliminary Tests.—Each of the Kalahari arrows was placed point downward over night in a test tube containing 20 cc. of sterile salt solution. Only traces of color and turbidity were imparted to the liquid. Five cubic centimeters of each suspension were injected into small guinea-pigs, weighing, respectively, 245, 252 and 272 grams. The last showed only a slight subcutaneous edema the following day, which quickly disappeared; the second showed no symptoms, but the first guinea-pig, though still alive after three hours, was dead the next morning (less than 15 hours) with extensive subcutaneous emphysema and hemorrhagic edema. The lungs were very white, the heart blood clotted, but the viscera were apparently normal. There were numerous long filaments and chains of gram-positive rods in smears made from the surface of the liver and the subcutaneous tissues, which were isolated and identified as *B. septicus* (1028). In short, none of the Kalahari arrows showed any evidence of being poisoned, but one of them evidently had pathogenic organisms upon it capable of fatally infecting a guinea-pig. Cultures were made from the tissues of this animal and also from all of the original suspensions; the findings, which are summarized below, show distinctly that these particular arrows would have been dangerous mainly for their infectious properties. The Ovachimba arrow was likewise soaked in salt solution, 30 cc. being necessary to cover the large iron point. The liquid became obviously rusty, but 5 cc. injected subcutaneously into a guinea-pig weighing 272 grams had no effect. There was thus no evidence of either strong poison or large numbers of infectious organisms upon the arrow. Cultures made from the suspension, however, yielded pathogenic bacteria, as will be shown.

In the case of the Heikum arrows sufficient material could be removed to be weighed. From the larger one 0.27 gram were diluted in 5.4 cc. of 0.85% salt solution, making a dirty dark brown suspension with considerable grit in it and a gray insoluble gummy residue. Microscopically, this was full of débris with no recognizable formed elements, bacterial or otherwise. One cc. of this suspension killed a 310 gram guinea-pig in less than 10 minutes, with violent spasms for about 1 minute before death. There was a slight subcutaneous hemorrhage and edema. The heart was stopped in marked systole and there had been

a severe gastric hemorrhage with rupture of the stomach wall. Otherwise the viscera were normal.

The remaining suspension was further diluted so that 1 cc. would contain 0.01 gram of the original poison. This amount killed a guinea-pig weighing 180 grams, by subcutaneous inoculation, in just 6 minutes with twitching and convulsions during the last four minutes. Necropsy performed at once showed localized subcutaneous hemorrhage, the heart stopped in systole, with the left auricle strongly congested, the lungs normal, the stomach strongly contracted but not ruptured, and marked peristalsis of the intestines. Another guinea-pig weighing 200 grams inoculated with the equivalent of 0.001 gram crude poison showed no sign of discomfort, either immediately or for several days following.

Cultures were made from the original suspension and yielded pathogenic organisms, as will be shown. It is obvious that their activity could be effective only under conditions where absorption of the poison was so slow, or the animal so large that some time would be required for its action. The fact that quite large animals, such as the wildbeeste, eland and giraffe, are killed by these small arrows suggests that such pathogenic bacteria may play a not inconsiderable rôle in their destruction.

Some of the dark hard material (0.105 gm.) was cracked from the shaft of the smaller Heikum arrow, just back of the point, and suspended in 10.5 cc. of 0.85% NaCl solution. Microscopically nothing could be recognized in the suspension except debris. Of this suspension, 1 cc. (0.01 gm.) killed a 235 gram guinea-pig by subcutaneous inoculation in about 5 minutes with terminal convulsions. There was a slight localized edema under the skin at the site of inoculation. The heart was stopped in systole and the lungs were normal. The stomach showed a superficial hemorrhage and was ruptured. Otherwise the viscera were microscopically normal. Subcutaneous doses of 0.5 cc. (0.005 gm.) and 0.3 cc. (0.003 gm.) killed small guinea-pigs in seven minutes with dyspnea and convulsions during the last two minutes. In each case slowing of the heart was noted prior to death. On immediate necropsy the findings were identical with those in the first animal except that the stomachs were not ruptured though there was gastric hemorrhage. A subcutaneous dose of 0.1 cc. (0.001 gm.) failed to kill a 290 gram guinea-pig within two hours but this animal was found dead and cold within fifteen hours. There was a slight local subcutaneous hemorrhage and emphysema. The lungs were slightly congested, the heart had stopped in systole, and the stomach was ruptured. No bacteria could be stained from the subcutaneous tissues or liver surface and a heart blood culture in brain medium was sterile. There was thus no evidence of infection in this animal. We have failed likewise to demonstrate any pathogenic organisms among the cultures from this arrow, the only one of the series giving this result.

Since poisoned arrows are used to kill game for food purposes and such poisoned or infected flesh, according to Dr. Cadle, is harmless when eaten by the bushmen, it was interesting to note that a small guinea-pig,

weighing 165 grams, fed 0.01 gram of the crude poison suspended in 1 cc. of water, by pipet, suffered no ill consequences whatever. This experiment was controlled by simultaneous inoculation of 0.005 gram into another animal with the fatal result described above and the fed pig itself two days later succumbed quickly to subcutaneous inoculation of 0.003 gram.

Pharmacologic Examination of the Heikum Poison.—As a preliminary step in the attempt to identify the active principle, tests were made for alkaloids. But the aqueous solutions were neutral to litmus and gave no precipitate with iodine, picric acid or mercuric-potassium iodide. There was thus no evidence for alkaloids.

Tests were then made for glucosides. The formation of a black precipitate when aqueous solutions of the poisons were heated with 10% AgNO_3 , and the formation of a greenish brown precipitate when 2 cc. of concentrated H_2SO_4 with a trace of FeCl_3 were added to 1 cc. of aqueous solution of the poison, showed the presence of reducing substances in the poison.¹³ Castellani and Chalmers'⁹ reference to the use of strophanthin and ouabain by African tribes led us to make special tests for these glucosides.

Two cubic centimeters of aqueous solution of poison from each arrow were layered upon 1 cc. of concentrated H_2SO_4 . At the surface of contact the acid layer became pink and the aqueous layer a dirty green. This is stated by Sollman¹⁴ to be a positive test for ouabain.

To 5 cc. of concentrated HCl containing a few crystals of resorcinol, 0.5 cc. of aqueous solution of poison were added and the mixture boiled. There was no marked change in color. According to Martindale and Westcott,¹⁵ a rosy color appears if strophanthin is present, but no change occurs in the presence of ouabain. The preliminary tests thus pointed to ouabain as the active principle.

Extraction and Purification of the Heikum Poison.—So little information was available as to methods of purification of ouabain that the procedures recommended by Fuller¹³ for the recovery of strophanthin were followed, although none of the tests had indicated strophanthin.

The crude poison scraped from the arrows had been partially dissolved and partially suspended in physiologic salt solution. These suspensions were evaporated to dryness over the boiling water bath at 94 C. The residue from the larger Heikum arrow (1026) was extracted three times with petroleic ether (chart 1). Evaporation of the petroleic ether solution (A_1) left an oily residue

¹³ Fuller: *Chemistry and Analysis of Drugs and Medicine*, 1920.

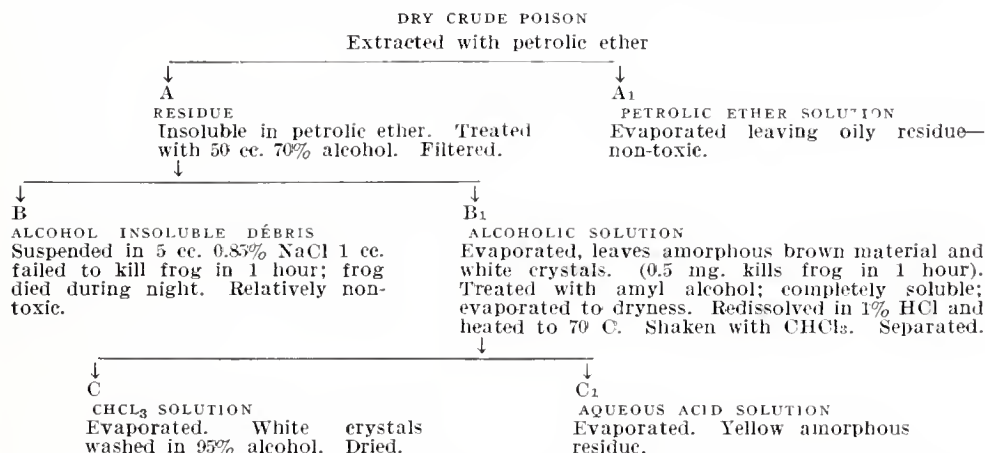
¹⁴ *Laboratory Guide in Pharmacology*, 1922.

¹⁵ *Extra Pharmacopeia*, 1925, 2.

which was insoluble in water and nontoxic for frogs. The petrolic ether insoluble residue (A) was treated with 50 cc. 70% alcohol, leaving an insoluble gritty debris (B). This was suspended in 5 cc. 0.85% NaCl and 1 cc. injected into the dorsal lymph sac of a frog, which lived more than an hour but died during the night. On evaporation of the alcoholic solution (B_1) there remained a mixture of brown amorphous material and white crystals, injection of 1 mg. of which, dissolved in 0.85% NaCl, into the dorsal lymph sac of a frog killed the animal within one hour with the heart stopped in systole. Further tests showed that 0.5 mg. would kill a frog weighing 43 grams within one hour. The dry residue also gave a reddish color with sulphuric acid (Sollman)¹⁴ but the resorcinol-hydrochloric acid test for strophanthin was negative.

The mixed amorphous and crystalline residue from the alcoholic solution (B_1) was then treated with three 10 cc. portions of amyl alcohol and found completely soluble. The amyl alcohol was removed by evaporation and the residue redissolved in 35 cc. of 1% HCl and heated to 70 C. The acid aqueous solution was then shaken with CHCl_3 in a separatory funnel. The chloroform (C) was separated and on evaporation left a mass of white crystals, which were washed in 95% alcohol and dried. The aqueous solution (C_1) was evaporated leaving a yellow amorphous residue (C_1).

CHART 1.—EXTRACTION AND PURIFICATION OF POISON FROM HEIKUM ARROW 1026



The residue of crude poison from the smaller Heikum arrow was handled differently (chart 2). It was first treated with 45 cc. 70% alcohol and allowed to stand for two hours with frequent shaking. It was then filtered and the filtrate (A) was tested for alkaloids with picric acid, iodine solution and mercuric potassium iodide with negative results. The alcohol insoluble debris (A_1) was not tested.

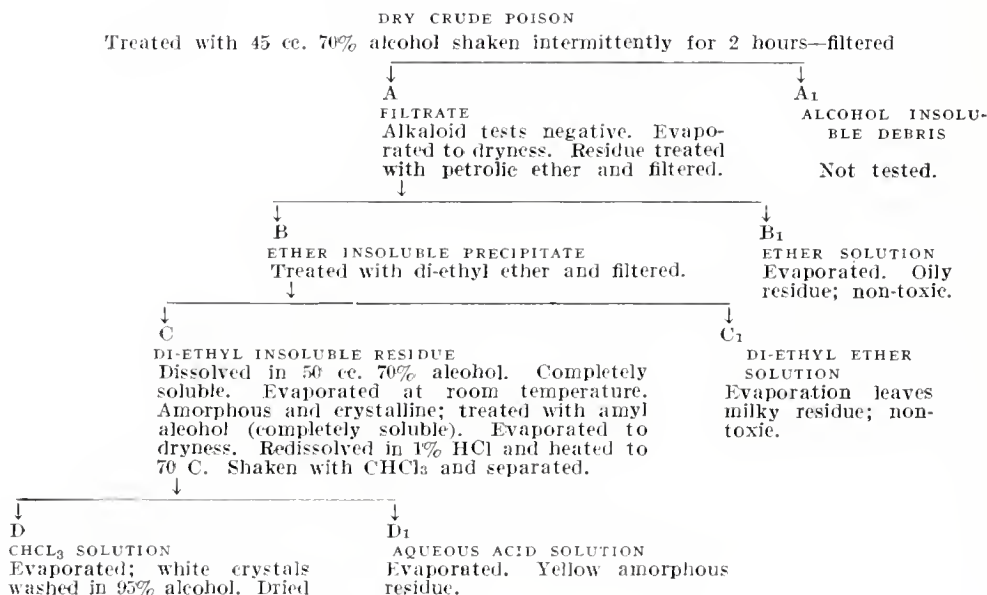
The filtrate (A) was then evaporated to dryness at room temperature and the residue treated with petrolic ether and filtered. The ether solution (B_1) was evaporated, leaving an oily nontoxic residue.

The ether insoluble precipitate (B) was treated with three portions of di-ethyl ether and filtered. The filtrate (C_1) left a milky residuc which proved to be nontoxic for frogs.

The di-ethyl ether insoluble residue (C) was again dissolved in 50 cc. of 70% alcohol. It was completely soluble. The alcohol was therefore evaporated, from there on following the same procedures as used for arrow 1026, (charts 1 and 2), and again separating two fractions, one white crystalline (D) the other yellow and amorphous (D_1).

Toxicity Tests of the Purified Fractions.—The method of Smith and McClosky¹⁶ was used to determine the MLD by intravenous injection into the musculocutaneous veins of pithed frogs, the time of observation being 1 hour, and the criterion of death, stoppage of the heart beat.

CHART 2.—EXTRACTION AND PURIFICATION OF POISON FROM HEIKUM ARROW 1027



The results for the crystalline fraction (C) of arrow 1026 are shown in table 1. In this test 2 mg. of crystals were dissolved in 100 cc. of 0.7% NaCl. It was found that the MLD per gram weight of frog was less than 0.00039 mg. but more than 0.00034 mg.

In another series with larger frogs the results were as shown in the second part of table 1. The solution was made to contain 2 mg. in 50 cc. of 0.7% NaCl. The larger frogs seemed to be somewhat more resistant as the MLD here was more than 0.00040 mg. but less than 0.00044 mg. per gram of frog.

The toxicity of the crystalline poison (D) from arrow 1027 was approximately the same as that from arrow 1026, as seen in table 2. These tests were made with 2 mg. of crystals dissolved in 50 cc. of 0.7% NaCl. The MLD per gram of frog was between 0.00033 mg. and 0.00042 mg.

¹⁶ Pub. Health Rep., 1925, Supplement 52.

The yellow amorphous material was tested in a similar manner on frogs. The tests for 1026 (2 mg. dissolved in 50 cc. 0.7% NaCl) are summarized in table 4. The MLD per gram of frog was more than 0.00074 mg. and less than 0.00079 mg.

TABLE 1
TOXICITY TITRATION OF CRYSTALLINE POISON FROM HEIKUM ARROW 1026

Weight of Frog, Gm.	Amount Injected, Cc.	Mg. per Gm.	Heart Beat at 1 Hour
15.5	0.3	0.00039	—
15.5	0.3	0.00039	—
21.0	0.5	0.00047	—
14.5	0.2	0.00027	+
19.0	0.1	0.00010	+
23.0	0.4	0.00034	+
SERIES WITH LARGER FROGS (Solution 2 mg. in 50 cc. 0.7% NaCl)			
30	0.55	0.00036	+
27	0.60	0.00044	—
32	0.65	0.00040	+
26	0.70	0.00038	+
27	0.75	0.00040	+
25	0.80	0.00046	—

TABLE 2
TOXICITY TITRATION OF CRYSTALLINE POISON FROM HEIKUM ARROW 1027

Weight of Frog, Gm.	Amount Injected, Cc.	Mg. per Gm.	Heart Beat at 1 Hour
20.0	0.05	0.00010	+
21.5	0.07	0.00010	+
34.0	0.15	0.00020	+
15.2	0.07	0.00020	+
18.6	0.08	0.00020	+
22.6	0.15	0.00020	+
18.3	0.09	0.00020	+
19.1	0.20	0.00042	—
17.5	0.20	0.00046	—
23.9	0.20	0.00033	+
21.4	0.25	0.00047	—
17.0	0.12	0.00028	+

TABLE 3
TOXICITY TITRATION OF AMORPHOUS POISON FROM HEIKUM ARROW 1026

Weight of Frog, Gm.	Amount Injected, Cc.	Mg. per Gm.	Heart Beat at 1 Hour
22.6	0.1	0.00018	+
28.4	0.1	0.00014	+
19.8	0.2	0.00040	+
20.1	0.2	0.00040	+
23.5	0.2	0.00034	+
20.2	0.3	0.00059	+
28.6	0.3	0.00045	+
21.3	0.4	0.00074	+
20.0	0.4	0.00080	—
27.3	0.5	0.00079	—
24.5	0.5	0.00081	—

Most of the amorphous poison from arrow 1027 having been used on cats, there was not enough to make comparative tests on frogs. The experiment on cats will now be described. These followed the method of Eggleston.¹⁷

¹⁷ J. Am. M. A., 1913, 61, p. 757.

Of the amorphous material (D_1) from arrow 1027, 2 mg. were dissolved in 40 cc. of 0.9% NaCl. A cat weighing 2.5 kg. was anesthetized with ether and a cannula inserted into the femoral vein through which the solution was allowed to run slowly. After the injection of 8.5 cc. of the solution, the animal began to show toxic symptoms and death occurred at the end of one hour and five minutes, 11 cc. containing 0.55 mg. of the poison having been injected. This gives an MLD of 0.22 mg. per kilo of cat (0.00022 mg. per gram).

A solution of amorphous material from the other arrow (1026) was tested on another cat (weight 1.6 kg.) with the result that the animal died after the injection of 8.7 cc. containing 0.435 mg. of poison, giving an MLD of 0.27 mg. per kilo of cat (0.00027 mg. per gram). The poison is about three times as poisonous for cats as for frogs according to body weight.

According to this experiment, the amorphous material from arrow 1026 has about the same toxicity as that from 1027, and according to the frog experiments, the amorphous material is about one half as toxic as the crystalline material.

Chemical Tests of the Poisonous Fractions.—The crystals from each Heikum arrow (1026 and 1027) were tested separately, but the results were the same for both.

The usual tests for alkaloids (picric acid, iodine and mercuric potassium iodide) were negative. Tests for cyanides and tannin were also negative.

Reduction tests with silver nitrate solution and with sulfuric acid containing ferric chloride were positive.

The Molisch test was negative with the crystals but positive after they had been hydrolyzed by boiling in 10% HCl for one hour, suggesting their glucosidal nature.

The sulphuric acid test for ouabain (Sollman)¹⁴ was positive but subsequent melting point and refraction tests showed that the poisonous crystals could not be ouabain. The resorcinol-HCl acid test for strophanthin was also negative.

A complete chemical analysis was impossible because of the small amount of material available.

The amorphous fractions from each arrow were also tested separately but with identical results for the two arrows.

The alkaloidal tests were negative, but the reducing tests with silver nitrate and with sulphuric acid containing ferric chloride were positive. The possibility of a double bond in the poison was suggested by Von Bayer's test (Holleman).¹⁸ The poison caused a marked reduction and formation of hydrated manganese dioxide. This test is not absolute however, as aldehydes react similarly with potassium permanganate.

The Molisch test was negative but after further portions of the poison were hydrolyzed by heating in HCl a positive Molisch test was secured, suggesting that the amorphous fraction also was a glucoside.

The HCl-resorcinol test for strophanthin was negative. The H_2SO_4 test for ouabain was positive.

Physical Tests of the Poisonous Fractions.—The crystals were dried at 110 C. They were then placed in capillary tubes and gradually heated in a sulphuric acid bath until they melted at 193 C.

¹⁸ Text Book of Organic Chemistry, 1915.

The optical properties of the crystals were determined by Dr. R. D. Crawford, Professor of Minerology and Petrology in the University of Colorado, who found two distinct types of crystals, one of hexagonal plates, showing an index of refraction of 1.484, parallel extinction, and anisotropism, the other of square plates, with an index of refraction of 1.544, parallel extinction, and isotropism.

It was impossible to identify either type of crystal for certain, with ouabain. For although a sample of ouabain (Eli Lilly & Co., 0.0005 grams in normal salt solution, Strophanthin Thoms from *Strophanthus gratus*) yielded on evaporation, crystals with an index of refraction between 1.533 and 1.553, thus resembling the second type above, they showed anomalous extinction, were oblong rectangular, and anisotropic.

The amorphous material fused and turned black at 119 C. Although the amorphous poison was not definitely resinous as in the case of the "euphorbion," mentioned by Perrot and Vogt,⁸ yet it resembled the resins in becoming less soluble in water after about 3 months' contact with air.

DISCUSSION OF WORK ON POISONS

It is obvious and unfortunate that we were unable definitely to identify the poison or poisons. All that can be said is that we recovered from the two Heikum arrows, small amounts of a crystalline poison and an amorphous poison. The crystalline poison consists of two types of minute crystals, which we were unable to separate, highly poisonous for cats and frogs, soluble in 70% alcohol, chloroform and water, insoluble in petroleic ether, and di-ethyl ether, not alkaloidal but probably glucosidal, resembling but not identical with ouabain.

It is well known that various glucosides have been found in arrow poisons, for several of the valuable cardiac remedies were first used by savage tribes. Ouabain (waba, wabajo, ouabaio) was described by Burton¹⁹ in 1856, having been studied as early as 1853 by Dr. F. S. Arnott and by Mr. R. Haines in the form of Somali arrow poison. According to Hilton, Fagge and Stevenson²⁰ and Fraser,²¹ Kirk first discovered the African arrow poison plant, *Strophanthus Kombe* in 1861 and recognized it as a powerful cardiac poison. Many years later Fraser and Mackenzie²² made a pharmacologic study of *S. sarmentosus* and its use as an arrow poison. The fact that both strophanthin and

¹⁹ Footsteps in East Africa, 1856. Reprinted, 1910.

²⁰ Proc. Roy. Soc., 1865, 15, p. 274.

²¹ Trans. Roy. Soc. Edinburgh, 1889, 35, p. 955.

²² Ibid., 1910, 47, p. 341.

ouabain stop the heart in systole suggests that the African arrow poison studied by Bolton²³ which stopped the heart in diastole must have been distinct, but Laidlaw's²⁴ tests of the poison on a Bini spear suggested strophanthin, although Laidlaw was unable to secure the drug in crystalline form. Further references can be found in Matthews²⁵ and Cushny.²⁶

Cultural Examinations—From each of the preliminary salt solution suspensions 1 cc. was inoculated into a tube of deep brain medium. Incubation of these tubes at 37 C. showed abundant gas production next day; they served as the primary cultures from which all subcultures and isolations were made.

Special presumptive tests were made for certain organisms.

Plates of eosin methylene blue agar streaked from the primary brain cultures failed to indicate any trace of *Bact. coli*, suggesting that either no fecal matter entered into the preparation of the arrows, or if so, this telltale organism had long since disappeared.

Cultures in milk (constricted tubes) indicated the presence of *B. Welchii* in only one, i. e., the large Heikum arrow. The avidity of this organism for lactose and the precedence of stormy fermentation of milk over the putrefactive activities of other bacteria in mixtures makes it seem quite certain that none of the other arrows contained *B. Welchii*.

Constricted tubes of chopped meat in glucose broth similar to those which Miss Peterson and I²⁷ found so useful for the detection of *B. botulinus* and *B. tetani* in soil, inoculated from the primary cultures, incubated for one week at 37 C., filtered through Mandler filters and injected into guinea-pigs, failed to show the presence of the toxins of these or any other microorganisms.

Subcutaneous inoculation of guinea-pigs with the supernatant fluid from the 24 hour primary cultures in brain medium, or, in the case of the poisonous Heikum arrows the third transplant to avoid by dilution any effect of the glucoside, showed that pathogenic bacteria were present in all but one of the arrows, i. e., the small Heikum arrow, in which none of the tests indicated their presence. It is unnecessary to give the results of these tests here in detail. Of the five animals showing lesions, two were dead on the day following inoculation, with the viscera protruding through the perforated wall and denuded skin of the abdomen. The other three showed the peculiar lesion following subcutaneous inoculation of *B. histolyticus*, i. e., a denuded area of skin, 2 to 3 cm. in diameter, which slowly healed. The fact that it was possible at first to isolate a pure culture of *B. histolyticus* from only one of the arrows was a matter of considerable concern and seemed to indicate either that this species succumbed to elective cultivation or that mixtures of other organisms might give rise to lesions closely resembling that of this species though no microorganism is known that will do so in pure culture. We were able ultimately, however, after considerable labor and manipulation to isolate *B. histo-*

²³ Proc. Roy. Soc. London, Series B, 1906, 78, p. 13.

²⁴ J. Physiol., 1910, 39, p. 354.

²⁵ Physiological Chemistry, 1920.

²⁶ Digitalis and Its Allies, 1925.

²⁷ J. Bact., 1924, 9, p. 201.

lyticus in pure culture from each mixture that gave the lesion characteristic for this organism in the preliminary presumptive tests for pathogenicity. In doing so we learned much concerning the technic that is necessary to recover this interesting species.

In view of the complex flora in each of the primary cultures, isolation was conducted separately for aerobes and anaerobes. The aerobes were isolated by streaking out upon blood agar plates. Isolation of anaerobes was attempted only after the heavily growing aerobes had been eliminated, nonsporulating forms by selective heating, sporulating forms by selective bacteriostasis.²⁸ The further details of isolation of the obligate anaerobes were essentially as outlined in previous publications²⁹ and consisted in repeated picking of isolated colonies from deep agar dilutions.

The isolation of *B. histolyticus* presented a peculiarly puzzling problem which had to be solved in a special manner. In the elimination of the heavily growing aerobes it was found to be an advantage to combine selective bacteriostasis with selective heating by boiling the inoculated broth culture for one minute (94 C. at Denver) before layering the broth above the marble seal with gentian violet. Of the 1:1000 dye solution, 0.5 cc. was added, making a concentration somewhat higher than I have formerly advocated, and I believe more efficacious. At any rate, it was possible in five cases to eliminate both sporulating and nonsporulating aerobes, excepting *B. histolyticus*, in the first attempt, though in one case five attempts were necessary. One always has to be careful not to allow the dye to seep below the seal.

We assumed that *B. histolyticus*, if still present, would be isolated along with the obligate anaerobes by picking dissimilar colonies from deep agar shake cultures in Burri tubes. Although numerous colonies were picked, none of them proved to be *B. histolyticus*. Yet the mother cultures from which the deep agar cultures were made, invariably gave evidence of the presence of this organism in pathogenicity tests in guinea-pigs by the characteristic sloughing of skin in subcutaneous inoculations and of muscle and skin in intramuscular injections.

We therefore utilized a property of *B. histolyticus* that has been generally overlooked, i. e., its ability to grow in delicate colonies upon meat infusion aerobic agar slants, which one of us (Hall³⁰) first described in 1923. We found that the mixed cultures freed from heavily growing aerobes by selective heating and selective bacteriostasis still gave a growth, so delicate in some cases that it could scarcely be detected with the naked eye, upon aerobic agar slants. Efforts to transfer single aerobic colonies from such slants were unsuccessful; we therefore undertook to enrich the cultures in *B. histolyticus* by successive transfers upon aerobic agar slants followed by the ordinary methods of an aerobic isolation of picking deep agar colonies. It was found, however, that even two or three aerobic transfers did not suffice to eliminate all of the obligate anaerobes, which apparently remained latent on the surface of the agar, for the deep isolation cultures failed to yield pure cultures of *B. histolyticus*, when the second or third aerobic agar culture was transferred back to deep brain and then into deep agar. Indeed several of the obligate anaerobes promptly overgrew *B. histolyticus*. *B. centrosporogenes* was isolated three times, *B. Novyi*, once, and *B. nonfermentans* n. sp., once, from deep brain cultures made from the third transfers upon aerobic agar slants.

We finally learned that the best method of isolating *B. histolyticus* from such mixtures was to continue the aerobic transfers until a subculture in glucose

²⁸ Hall: J. Am. M. A., 1919, 72, p. 275.

²⁹ Hall: J. Infect. Dis., 1920, 27, p. 576; 1922, 3, p. 445.

³⁰ Proc. Soc. Exper. Biol. & Med., 1923, 20, p. 501.

broth failed to produce acid. Such cultures always proved in this study to be pure *B. histolyticus* as checked by the identical characters of well isolated colonies in deep agar. Five aerobic agar slant transfers usually sufficed to secure glucose broth subcultures in which no acid production occurred but it is quite possible that other combinations than those with which we worked would require more. We particularly caution against the assumption that the method outlined is an infallible means of securing pure cultures of *B. histolyticus*. It would probably fail in the presence of *B. tertius* and other micro-aerophilic aerobes.³¹ *B. tertius* was not encountered in any of the arrow material. The method is rather to be regarded as one for enrichment, to be followed by the usual procedures for anaerobic isolation. All of our cultures of *B. histolyticus* were reisolated at least twice and rechecked by animal tests before their purity was assumed.

The following were isolated in pure culture and identified. The numbers following the specific names refer to our own laboratory records. Those which are starred are pathogenic for guinea-pigs, showing the lesions characteristic for the species.

Kalahari Arrow 1

Streptococcus mitis (1022 b)
B. centrosporogenes (1022 f)
 **B. histolyticus* (1031 b)
B. bifermentans (1031 d)

Kalahari Arrow 2

B. centrosporogenes (1023 x)
 **B. histolyticus* (1091)

Kalahari Arrow 3

Streptococcus mitis (1024 b)
Staphylococcus albus (1024 c)
 **B. histolyticus* (1024 f)
 **B. septicus* (1028) †

† Isolated from heart blood of guinea-pig dead after subcutaneous inoculation of 5 cc. sterile salt solution in which arrow had soaked overnight. The animal showed extensive edema, emphysema and some hemorrhage. Failure to recover this species directly from the primary culture of the arrow raises a question as to whether it may not have come from the animal.

**B. Novyi* (1033A)

B. centrosporogenes (1033 B)

Ovachimba Arrow

B. centrosporogenes (1025 x)
B. nonfermentans n.sp. (1025 y)
 **B. histolyticus* (1076)

Large Heikum Arrow

B. nonfermentans n.sp. (1026 x)
B. centrosporogenes (1045)
 **B. Welchii*—type 4—(1048)
 **B. histolyticus* (1079)

Small Heikum Arrow

B. subterminalis n.sp. (1072)

In addition to the above, every arrow had "hay bacilli" upon it. Several species were isolated, but we have been unable to identify any of these satisfactorily owing to the lack of suitable classificatory keys; we call attention to the need for more specialized taxonomic studies of the sporulating aerobic bacilli. While these organisms, excepting the anthrax bacillus, are nonpathogenic in pure cultures, they may be of some importance in mixed infections. *B. anthracis* did not appear among the organisms isolated from the arrows.

Two species of obligate anaerobes were recovered which apparently have not been previously described. Though differing, one from the other, they are alike in failing to ferment any of the common carbohydrates. Using this characteristic as a clue, we failed to find any descriptions in the available literature of nonfermentative anaerobes with the other properties possessed by these.

³¹ Hall and Matsumura: *J. Infect. Dis.*, 1924, 35, p. 502.

B. nonfermentans n. sp. (1025 x) was isolated from the Ovachimba arrow. It is a motile gram-positive rod, forming central or excentric oval spores that do not swell the rods. The rods occur singly or in pairs, rarely in short chains. The relation to oxygen is that of an obligate anaerobe, no visible growth ever occurring upon the surface of aerobic blood or meat infusion agar. It grows readily in brain medium, with slight turbidity in the overlying liquid, a little gas production, and slow digestion with discoloration. Cultures in brain medium with added metallic iron show the marked black discoloration characteristic of iron sulfide within 2 or 3 days. Cultures more than 4 months old in brain medium do not show any formation of crystals. Well isolated colonies in deep 1% meat infusion agar cultures are irregular in shape and slightly granular. Glucose broth cultures in constricted tubes show turbidity without gas production. There is no acid production in glucose, levulose, galactose, or maltose; such cultures are always either neutral or slightly alkaline to bromthymol blue. There was no evidence of change in milk cultures during nine day's observation. Blood agar slants, anaerobic by alkaline-pyrogallol, produce delicate nonhemolytic colonies. Gelatin cultures become slightly turbid, with blackening of the precipitate and slow liquefaction (4 days). The species is non-pathogenic for guinea-pigs by subcutaneous inoculation of glucose broth and brain cultures.

An organism (1026 x) was also recovered from the large Heikum arrow identical with *B. nonfermentans* except that it failed to liquefy gelatin, possibly due to failure of growth though heavy inoculations were made. We do not feel justified however in setting up a new and separate species, and prefer for the present to classify the two together, pending the possible collection of further strains and a more detailed study of nonfermentative anaerobes with central spores of which *B. nonfermentans* seems to be the first species described.

B. subterminalis n. sp. (1072) was recovered from the small Heikum arrow, being apparently the only anaerobe present. It is a motile gram-positive rod, forming oval subterminal spores that distinctly swell the rods. The rods occur singly or in pairs, rarely in short chains. No growth ever occurs on the surface of aerobic blood or meat infusion agar slants; it is an obligate anaerobe. It grows readily in brain medium with slight turbidity of the overlying liquid, a little gas formation and slow digestion of the protein. Iron brain cultures produce black iron sulphide in 2 or 3 days. Old cultures do not produce any crystals such as tyrosine. Deep 1% agar colonies are opaque, compact, biconvex,

or lobulate, discs. Glucose broth becomes turbid but no gas or acid is produced in glucose, levulose, galactose, maltose, or lactose.

Milk, curiously enough, slowly coagulates (2 or 3 days), with mild acidity and gas production and eventually (8-18 days) complete liquefaction of the coagulated casein. We have been unable to explain the anomalous action in milk, except upon the hypothesis that there may be a carbohydrate in milk other than those failing to ferment in our tests. The result with milk was obtained repeatedly.

Anaerobic blood agar slants show delicate colonies, mildly at first, later actively, hemolytic. Gelatin is slowly liquefied with slight turbidity and blackening of the sediment. Several subcutaneous inoculations of glucose broth and brain medium cultures into guinea-pigs failed to indicate any pathogenicity.

B. subterminalis may be distinguished from *B. nonfermentans*, and from the nonfermenting bacilli that form terminal spores, by its different morphology, its action in milk, and its hemolytic action on blood agar; it may be distinguished from *B. histolyticus* by its failure to grow aerobically, its failure to produce tyrosine, and its lack of pathogenicity.³²

DISCUSSION OF BACTERIOLOGIC STUDY

Any bacteriologic study of objects that have been handled as these arrows have, must submit to the criticism that the organisms recovered may have been derived from external sources. We really have no satisfactory standard of comparison, such for example, as a similar study of miscellaneous sticks might offer. It seems doubtful, however, whether the sporulating anaerobes and particularly pathogenic forms such as described, are to be found equally on objects not definitely subjected to contamination, although doubtless the filthy habits of the bushmen would be such as to contaminate his utensils of whatever nature with organisms of the soil. This is probably the true explanation of the origin of the bacteria upon the arrows. The organisms found are typical of the soil flora and it was quite surprising that there was not some evidence of fecal pollution. It was equally surprising not to find *B. tetani* and *B. botulinus*, considering the well known frequency of these forms in the soil.

The essential lesson seems to be that arrow wounds are potentially similar to shrapnel wounds in their pathogenic effect, except that if the arrow happens to be a poisoned one, there are three possibilities for death instead of two, first, by direct trauma, second, by action of the poison, third, by disease processes set up by pathogenic bacteria.

SUMMARY

Attention is again called to the general recognition that wounds by arrows, poisoned or otherwise, are likely to be infected wounds. It is really surprising that so few bacteriologic studies of such wounds have been made, comparable with those on bullet and shrapnel wounds and that no bacteriologic studies of poison arrows are recorded anywhere in the available literature.

The present study deals with six African bushman arrows obtained by Dr. C. E. Cadle during the Denver African Expedition of 1925 from the Kalahari, Ovachimba and Heikum tribes. The probably complex nature of the poison is suggested. Only the Heikum arrows had poison upon them. There was no evidence of alkaloidal poisons. The poison was separated into amorphous and crystalline fractions, the former about one-half as toxic as the latter. The crystalline fraction was fatally toxic for frogs in doses of 0.00039 to 0.00044 mg. per gram weight of frog. Both fractions behaved alike, killing guinea-pigs, cats and frogs by stopping the heart in marked ventricular systole. The poisons could not be identified with any known drugs but resembled the glucoside ouabain in some respects. Two kinds of crystals were noted but the small amount available precluded separation.

All of the arrows, except the smaller Heikum arrow had pathogenic bacilli upon their points in addition to the nonpathogenic "hay bacilli"; the obligate anaerobes (*B. centrosporogenes*, *B. bifermentans*, *B. sporogenes*, *B. nonfermentans* n. sp., *B. subterminalis* n. sp.) and the aerobes (*Staphylococcus albus*, *Streptococcus fecalis*, and *Streptococcus mitis*) occurred as indicated. The pathogens were *B. histolyticus*, *B. Novyi*, *B. septicus*, and *B. Welchii*. All of the infected arrows had *B. histolyticus* on them, and the special difficulties encountered in the isolation of this organism in pure culture from mixtures led to the development of a special technic for this purpose, utilizing the fact that *B. histolyticus* is a facultative aerobe, rather than an obligate anaerobe as generally supposed.

A STUDY OF LEPTOSPIRA ICTEROHAEMORRHAGIAE

VIRGINIA LANGWORTHY AND ANNA C. MOORE

From the Division of Laboratories and Research, New York State Department of Health, Albany

During the fall of 1921 and winter of 1922 cases occurred in various localities throughout New York State, with a characteristic group of symptoms, the most striking of which was jaundice. The symptom complex and grouping of cases, suggesting an infectious disease with jaundice as a characteristic symptom, led to the diagnosis of "infectious jaundice" under which caption a preliminary report of the investigation of this outbreak was published.¹ At that time the investigation had not progressed to a point where it was possible to draw definite conclusions.

It is the purpose of this paper to summarize the results of further studies—to bring out the points in common between certain epidemics of so-called infectious jaundice that have been described in the literature and the outbreak in New York State, to present evidence which tends to exclude any etiologic relationship between *Leptospira* (*Spirochaeta*) *icterohaemorrhagiae*² and the latter outbreak, and to record the observations made in our experimental studies which have established the identity of the spirochete isolated from Albany rats with *Leptospira icterohaemorrhagiae*.

Jaundice is a symptom and not a disease. It has long been recognized as an accompaniment of certain acute infections, such as yellow fever, relapsing fever and malaria or even pneumonia. Its association, however, with diseases, which although apparently infectious, were of undetermined etiology and pathologically not well defined, has led to their being characterized as "infectious jaundice," the loose employment of the term bringing into this category such dissimilar conditions that the connection between the diseases described in historical accounts is quite uncertain.

Received for publication, April 11, 1927.

¹ Wadsworth, A. B.; Langworthy, H. V.; Stewart, F. C.; Moore, A. C., and Coleman, M. B.: *J. Am. M. A.*, 1922, 78, p. 1120.

² Inada, R.; Ido, Y.; Hoki, R.; Kaneko, R., and Ito, H.: *J. Exper. Med.*, 1916, 23, p. 377. Noguchi, H.: *Ibid.*, 1918, 27, p. 575.

Throughout descriptions of military campaigns³ are accounts of a mild type of disease characterized by jaundice and involving large numbers, in some epidemics, hundreds and even thousands of persons. Outbreaks similar in nature but less extensive have occurred in the civil population also; a survey of those reported in the United States, since 1812, has been included in the recent study by Blumer.⁴ In these epidemics the disease has in general conformed to the epidemic catarrhal jaundice" described by Osler and McCrae;⁵ the febrile reaction was slight and of short duration, the principal symptoms, aside from jaundice, being headache, anorexia, abdominal discomfort and in some cases, acute gastrointestinal disturbances. The incidence of such symptoms as enlargement of the liver and the spleen has varied. In certain epidemics the occurrence of cases having other characteristic symptoms, but lacking jaundice, has been noted.⁴ The important bacteriological investigations have been made only during and since the recent war. The examination of bile, obtained with a duodenal sound, revealed the presence of organisms of the colon group in a considerable proportion of the cases studied by Hatiegan⁶ in the German army, and the presence of paratyphoid bacilli in those studied by Frugoni and his associates⁷ in the Italian army. Paratyphoid bacilli were found also in over half the blood cultures made in jaundice cases during the febrile stage, by Sarrailhé and Clunet⁸ at the Dardanelles. An etiologic relationship of the paratyphoid bacillus to epidemic jaundice was not indicated, however, by the results of the English (Archibald and his associates and Martin³) at the Dardanelles, nor by those of Brugsch and Schürer⁹ in the German army. In regard to the mode of transmission as well as the etiology the evidence is inconclusive. Certain of these epidemics (Benczúr, Paiseau, Archibald, Martin³) occurred at a season when insect transmission might be indicated, but some of the most extensive (Woodward and Fröhlich³) and indeed the majority of those analyzed by Blumer⁴ occurred during the cold months. In some outbreaks infection appears to have developed from a common source, such as contaminated food or water; in others, by more direct contact. The only outstanding features common to these outbreaks clinically like "epidemic catarrhal jaundice" are the mildness and the tendency to involve large numbers of cases. Thus, the evidence suggests that so-called infectious jaundice is not a disease of specific bacterial etiology but is induced by different pathogenic species, which give rise to infections that under certain conditions involve the liver with symptoms of jaundice.

³ Woodward, J. J.: Outlines of the chief camp diseases of the United States armies; a practical contribution to military medicine as observed during the present war. 1863, p. 193. Fröhlich, C.: *Deutsch. Arch. f. klin. Med.*, 1879, 24, p. 394. Benczúr, J.: *Deutsch. med. Wchnschr.*, 1916, 42, p. 482. Paiseau, M. G.: *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1916, 40, p. 60. Archibald, R. G.; Hadfield, G.; Logan, W., and Campbell, W.: *J. Roy. Army Med. Corps.*, 1916, 26, p. 695. Martin, C. J.: *Brit. M. J.*, 1917, 1, p. 445. Chambers, G.: *J. Roy. Army Med. Corps.*, 1917, 29, p. 108.

⁴ *J. Am. M. A.*, 1923, 81, p. 353.

⁵ Boggs, T. R., in Osler and McCrae: *Modern medicine: its theory and practice in original contributions by American foreign authors*, 1913, 1, p. 1014.

⁶ *Wiener klin. Wchnschr.*, 1919, 32, p. 956.

⁷ Frugoni, C., and Cannata, S.: *Sperimentale*, 1916, 70, p. 25. Frugoni, C.; Gardenghi, G., and Ancona, G.: *Ibid.*, 1916, 70, p. 587.

⁸ *Lancet*, 1916, 190, p. 664.

⁹ *Berlin. klin. Wchnschr.*, 1919, 56, p. 601.

Likewise included in the category of "infectious jaundice," but apparently distinct from the foregoing epidemic forms, is the acute febrile disease, which, since 1886, has been known as Weil's disease.¹⁰ Although Weil's critical clinical analysis was the stimulus for the studies of numerous European observers, the same disease very probably was described by other writers before Weil. In clinical features Weil's disease strongly resembles the "bilious typhoid" described by Griesinger¹¹ in Egypt as well as the "typhus hepaticus" of Mathieu.¹² Typically Weil's disease is accompanied by marked general symptoms, high fever, intense muscular pains, nervous symptoms, enlargement of the spleen, jaundice, hemorrhages and nephritis. Although cases lacking jaundice or other of the usual symptoms have been recognized when occurring in outbreaks with those of the classical type,¹³ the fact of the etiology being unknown precluded the recognition of atypical forms occurring sporadically. Data as to its prevalence are, therefore, incomplete. Considering only the cases conforming to Weil's description, certain epidemiologic facts are outstanding: the disease has not occurred in extensive epidemics but only in sporadic cases or strictly localized outbreaks; transmission has not been observed to occur by personal contact but has appeared to depend upon particular conditions, as evidenced by its occurrence among butchers and sewer workers¹⁴ and its outbreak in military garrisons in association with the swimming baths;¹⁵ cases have been reported at all seasons of the year, but have been most numerous during the summer months.

The first notable advance toward classification of infections characterized by jaundice was made in 1914, when Inada and his associates,² discovered the spirochetal origin of a severe febrile jaundice endemic in Japan, and thus supplied a basis for differentiating one specific infection from the heterogeneous groups. This spirochetal infection, which, from its clinical features had been considered Weil's disease, was named by the Japanese, *spirochaetosis icterohaemorrhagica*. Its identity with cases described as Weil's disease in Europe during the World War was shortly indicated by the independent etiologic studies of the German investigators, Hübener and Reiter,¹⁶ and Uhlenhuth and Fromme,¹⁶ which demonstrated the presence of the spirochete in their cases and confirmed in all essentials the findings of the Japanese. Following the publication of the Japanese discoveries in English, the infection was likewise demonstrated among allied troops by English,¹⁷ French¹⁸ and Italian¹⁹ investigators.

The causative organism, named *Spirochaeta icterohaemorrhagiae* by Inada,² but since placed by Noguchi in the genus "*Leptospira*," is ordinarily present in the blood stream, in human cases, during the first week only. Even during the acute stage, the leptospiras are rarely so numerous in the blood as to be

¹⁰ Deutsch. Arch. f. klin. Med., 1886, 39, p. 209.

¹¹ Arch. f. physiol. Heilkunde, 1853, 12, pp. 29 and 309. In Bamberger (and others): Handbuch der spec. Path. u. Therapie, 1864, 2, p. 285.

¹² Rev. de Medecine, 1886, 6, p. 633.

¹³ Hecker, A., and Otto, R. W.: Beiträge zur Lehre von der sog. "Weil'schen Krankheit." Klinische und ätiologische Studien an der Hand einer Epidemic in dem Standorte Hildesheim während des Sommers, 1910, 1911.

¹⁴ Fiedler, A.: Deutsch. Arch. f. klin. Med., 1888, 42, p. 261; *ibid.*, 1892, 50, p. 232. Stirl, O.: Deutsch. med. Wchnschr., 1889, 15, p. 798.

¹⁵ Med. Klinik, 1915, 11, pp. 1202 and 1264.

¹⁶ Deutsch. med. Wchnschr., 1915, 41, p. 1275; *ibid.*, 1916, 42, pp. 1, 131 and 1282.

¹⁷ Stokes, A.; Ryle, J. A., and Tytler, W. H.: Lancet, 1917, 192, p. 142.

¹⁸ Martin, L., and Pettit, A.: Presse méd., 1916, 24, p. 569.

¹⁹ Bravetta, E.: Policlinico, 1918, 25, p. 485.

found by direct microscopic examination. Their presence may be demonstrated indirectly, however, by animal inoculation; the injection into a guinea-pig of 2 to 5 cc. of blood containing leptospiras generally produces a fatal infection, characterized by symptoms similar to those of the human subject. The leptospiras are usually numerous at death in the blood and organs, particularly the liver, of an experimentally infected guinea-pig. They are found in the tissues of fatal human cases also, although rarely in large numbers. By the use of a fluid or semisolid medium containing a suitable animal serum, the organisms can be recovered in pure culture from the body fluids or organs of infected animals. The infection may be transmitted from diseased to healthy animals by the injection of blood or of organ emulsions containing leptospiras, or of a pure culture. The spirochetes have been found in all excretions but are discharged chiefly in the urine, the period of greatest elimination in human cases being the third week, coincident with the appearance of specific immune substances in the blood. The experimental evidence has thus fully established the etiologic relationship of the organism to the disease; moreover, the pathological changes do not differ fundamentally or essentially in the susceptible animal and in man.

The pathological anatomy of spirochaetosis icterohaemorrhagica has been given special attention by Kaneko,²⁰ who has reviewed European work on this subject and has added an important contribution through his analysis of a large series of cases in Japan. The pathological changes as described by him and others manifest a general, severe, toxic injury.

At necropsy the principal findings are generalized icterus, numerous hemorrhages in the skin, mucous membranes, serous surfaces and various organs, particularly in the lungs, and degenerative changes, especially in the kidneys, the liver and the skeletal muscles.

The changes in the kidney consist of degeneration of the epithelial cells, particularly in the convoluted tubules, associated with hemorrhages and cellular infiltration of the interstitial tissue, with a slight amount of connective tissue proliferation. Hemorrhage into the tubules has been noted, but an acute hemorrhagic nephritis is rare. The glomeruli are almost never involved. The findings in the liver vary according to the stage at which death occurs. In the early stage there is degeneration and some dissociation of the liver cells; there are also hemorrhages and cellular infiltrations particularly in the periportal region. In the later stages, in addition to degeneration and disassociation of the liver cells, there is bile stasis in the intralobular bile sinuses and deposition of bile pigment in the liver cells. The interlobular ducts and the extra hepatic bile passages, however, remain patent. Marked regenerative changes of the liver columns have been noted in the later stages in certain cases. The lesions of the skeletal musculature, which are most severe in the calves, consists of foci of hyaline degeneration, involving individual muscle fibres, accompanied by cellular infiltration, small hemorrhages, and proliferation of the nuclei of the sarcolemma.

The characteristic blood changes are a moderate polynuclear leukocytosis, which occurs at the beginning, and a secondary anemia which is apparent in the later stages or during convalescence.²¹

²⁰ Kaneko, R.: Über die pathologische Anatomie der Spirochaetosis icterohaemorrhagica Inada (Weilsche Krankheit). Vienna, Rikola, 1922.

²¹ Garnier, M., and Reilly, J.: Arch. de Med. exper. et d'Anat. path., 1916-17, 27, p. 609; Gudzent: Deutsch. med. Wchnschr., 1917, 43, p. 69; Luger, Alfred: Ibid., p. 747; Klieneberger, C.: Berlin. klin. Wchnschr., 1917, 54, p. 676; Deutsch. Arch. f. klin. Med., 1918, 127, p. 110.

The incubation period is ordinarily about seven days. The onset is typically abrupt, with chills and high fever. The main symptoms of the acute stage are extreme prostration, conjunctival congestion, headache, severe muscular pains, gastrointestinal disturbances and albuminuria. Jaundice usually appears by the fifth day and reaches its greatest intensity during the second week, when the initial symptoms ameliorate and the more pronounced manifestations are hemorrhages, nephritis and cardiac weakness. Death most commonly occurs in this stage. The initial pyrexia usually lasts from seven to ten days. After an afebrile period of variable length there, is in many cases, a recrudescence of fever. Convalescence usually begins in the third week.

From a comparison of the clinical features of the disease given by Inada²² with those described by representative European authors,²³ it is evident that in European cases icterus was a less constant symptom, the disease was generally less severe and the mortality rate lower (about 4% as compared with 32%).

Since the use of precise laboratory methods for proof of this infection, strikingly atypical clinical forms have come to light. The febrile reaction may be so slight and other symptoms so mild as to suggest so-called catarrhal jaundice²⁴ or icterus may be lacking and respiratory or neurological symptoms so prominent that the clinical picture simulates that of influenza²⁵ or meningitis.²⁶

Except in Japan, where spirochaetosis icterohaemorrhagiae is endemic, and where according to Tohyama,²⁷ as many as 5000 cases have occurred in a single year, authentic cases, that is, cases in which *L. icterohaemorrhagiae* has been demonstrated, have appeared only sporadically or in localized outbreaks. While the great majority of the cases in Japan have occurred among persons working in damp soil or in wet mines, food handlers, as well, have shown a special incidence of the disease. During the European war it appeared principally among troops in the trenches. The few undoubted cases reported since then include a small group among coal miners in Scotland²⁸ and sporadic cases in various European countries: one occurred in a steward on a ship,²⁹ six in persons who had come in contact with manure, sewage or sewage-polluted water,³⁰ and two in men who had worked in places infested by rats.³¹ In the United States, the only case known to have developed except through a laboratory infection is that

²² J. Exper. Med., 1917, 26, p. 355.

²³ Schott, E.: München. med. Wchnschr., 1916, 63, p. 1509; Reiter, H.: Ztschr. f. klin. Med., 1919, 88, p. 459; Dawson, B., and Hume, W. E.: Quart. J. Med., 1916-17, 10, p. 90; Ryle, J. A.: Ibid., 1920-21, 14, p. 139; Pagniez, and others: Bull. et mém. Soc. méd. d. hôp. de Paris, 1917, 41, p. 1181; Nolf, P., and Firket, J.: Arch. Méd. Belges, 1918, 71, p. 380; Garnier, M.: (French supplement) Lancet, 1919, 197, p. 1145.

²⁴ Garnier, M., and Reilly, J.: Bull. et mém. Soc. méd. d. hôp. de Paris, 1917, 41, p. 69.

²⁵ Goebel: Med. Klinik, 1916, 12, p. 381.

²⁶ Costa, S., and Troisier, J.: Bull. et mém. Soc. méd. d. hôp. de Paris, 1916, 40, p. 1928.

²⁷ Japan Med. World, 1924, 4, p. 193.

²⁸ Gulland, G. L., and Buchanan, G.: Brit. Med. Jour., 1924, 1, p. 313.

²⁹ Rimmer, R.: Brit. M. J., 1917, 1, p. 453.

³⁰ Manson-Bahr, P.; Wenyon, C. M., and Brown, H. C.: Lancet, 1922, 203, p. 1056; Schürer, Johannes: Med. Klinik, 1922, 18, p. 533; de Lavergne, V., and Perrier, P.: Bull. et mém. Soc. méd. d. hôp. de Paris, 1925, 49, p. 513; Goudsmit, J.; Hammer, E., and Wolff, J. W.: Nederl. Tijdschr. v. Geneesk., 1925, 1 (69), 430 (Abst., Centralbl. f. Bakt. 1, Ref., 1926, 82, p. 502); Schüffner, W., and Ruys, C.: Nederl. Tijdschr. v. Geneesk., 1925, 2 (69), p. 1020 (Abstr., Brit. M. J., 1925, 2, p. 49).

³¹ Schüffner and Ruys³⁰; Enneking, J.: Nederl. Tijdschr. v. Geneesk., 1926, 1, p. 1063 (Abstr., Brit. M. J., 1926, 1, p. 101).

of a man who worked and slept in a restaurant where rats were numerous.³² Although the diagnosis in this case was not confirmed by animal inoculation, the clinical and pathological findings were typical, and organisms morphologically resembling *L. icterohaemorrhagiae* were seen in sections of the liver, heart and kidney, prepared by Levaditi's method. There is no evidence of the infection of different individuals from a common supply of food or drinking water, nor transmission by personal contact. Insect transmission of spirochaetosis, although suggested by its special prevalence during the summer months, and reported by Reiter,³³ and Uhlenhuth and Kuhn³⁴ to have been accomplished experimentally with certain varieties of flies, has not actually been established. The factor which seems to be essential, in the development of spirochaetosis, is exposure to insanitary surroundings, more particularly exposure of the skin.

The probable source of the leptospira in damp and insanitary places was discovered in 1917, when the Japanese worker Ido and his associates³⁵ found an apparently identical organism to be carried by wild rats, this organism being present in the rat's kidneys and excreted in the urine. Infected rats have since been found to be common in practically all parts of the world, not only where outbreaks have occurred, but also in localities where the disease is unknown. As the leptospira is sensitive to drying and sunlight, and loses its virulence when cultivated outside the animal body, the infrequency of recognized human infection, in spite of the wide distribution of the inciting agent, is attributed to the brief viability of the organism after excretion from the rat. It appears that under particularly favorable conditions of saprophytic existence, however, the virulence may be retained for at least a limited period, for Buchanan³⁶ has recently discovered organisms identical in morphology and pathogenic properties with *L. icterohaemorrhagiae*, growing in slime in the mines of East Lothian where an outbreak of spirochaetosis had occurred. Whether the leptospires can retain their virulence indefinitely in such a natural habitat, or whether the rat is essential to maintaining a supply of virulent leptospires has not been determined.

Since the diagnosis of spirochaetosis icterohaemorrhagica has been placed on a precise basis, through the knowledge of its etiology, it has become evident that the clinical and epidemiologic features of this infection also differentiate it, to a certain extent, from many other conditions which have been described as infectious jaundice.

Spirochaetosis icterohaemorrhagica is an acute infection, characterized by a severe toxic injury with febrile reaction, the special manifestations of which are jaundice, hemorrhage and albuminuria. Spirochaetosis, in its typical clinical form, appears to be identical with the European Weil's disease. While individual cases of spirochaetosis may vary so markedly from the classical type as to be clinically indistinguishable

³² McDowell, E. S.: N. Y. State J. Med., 1925, 25, p. 19.

³³ Deutsch. med. Wchnschr., 1917, 43, p. 552.

³⁴ Ztschr. f. Hyg. u. Infektionskrankh., 1917, 84, p. 517.

³⁵ J. Exper. Med., 1917, 26, p. 341.

³⁶ Brit. M. J., 1924, 2, p. 990.

from other forms of jaundice or various other infections, no record has been found of an outbreak consisting exclusively of such mild or atypical cases.

Epidemiologically, spirochaetosis is distinguished by the fact that it is apparently "noncontagious;" it usually occurs sporadically or in localized outbreaks of limited extent, and is consistently associated with a damp or insanitary environment. These facts support the supposition that the rat as carrier is the main source of the infection. From the infrequency of the disease, moreover, in spite of the prevalence of rats that are carriers, it appears that transmission to the human subject occurs only under special conditions, such as contact with freshly excreted rat urine, or with contaminated water or soil favorable to the survival of the leptospira. In its tendency to appear sporadically, as well as in its restriction to damp or insanitary places, spirochaetosis resembles Weil's disease. It differs notably, however, from the jaundice of apparently infectious origin which has occurred in extensive epidemics.

Comparison of the Outbreak in New York State with Spirochaetosis icterohaemorrhagica.—Clinically, the disease in New York State was milder than spirochaetosis. The febrile reaction was slight; albuminuria and epistaxis, rare; and conjunctival congestion, stressed by the Japanese (22) as diagnostic of spirochaetosis, was likewise infrequent. Epidemiologically, the outbreak was much more extensive than those of spirochaetosis in countries with modern sanitary conditions. The disease further differed from spirochaetosis in its apparent "contagiousness"¹—multiple cases were frequent in families and in schools; cases developed, too, in the immediate families of children attending the same schools. The cases, moreover, were not restricted to insanitary surroundings. Although the presence of rats was noted in certain places where the disease occurred, the majority of the persons affected were living under conditions such as to make contact with fresh excretions of rats extremely improbable.

A consideration of the clinical and epidemiologic distinctions between the disease in New York State and spirochaetosis, together with the negative results of animal inoculations and immunological tests previously reported,¹ indicate that *L. icterohaemorrhagiae* had no etiologic relationship to this outbreak. Exclusion of spirochaetosis leaves the diagnosis of the epidemic in New York quite indeterminate.

Leptospira icterohaemorrhagiae Infection in Wild Rats.—Parallel with the search for *L. icterohaemorrhagiae* in the cases of jaundice, a study was made of the prevalence of the infection in rats, and of the immunological reactions associated with the carrier state.

Examinations for *L. Icterohaemorrhagiae*: The rats were captured alive and chloroformed, and in most instances blood was obtained by heart-puncture for serological tests before the rat was killed. Immediately after death the kidneys of each rat were removed aseptically, ground finely with sterile glass in a mortar, and emulsified in about 10 cc. of physiologic salt solution. One cc. of the emulsion was then injected intraperitoneally into each of two (and in some instances three) guinea-pigs, and if urine was obtainable that was injected into a guinea-

TABLE 1
RESULTS OF EXAMINATIONS OF WILD RATS FOR LEPTOSPIRA ICTEROHAEMORRHAGIAE

		Examination by Inoculation of Guinea-Pigs with Rat Urine or Kidney Emulsion							
		Infection Characteristic of <i>L. icterohaemorrhagiae</i> in Guinea-Pigs				No Evidence of Infection with <i>L. icterohaemorrhagiae</i> in Guinea-Pigs			
		Dark-Field Examination of Rat Urine or Kidney Emulsions for <i>Leptospira</i>				Dark-Field Examination of Rat Urine or Kidney Emulsions for <i>Leptospira</i>			
Source of Rats	Total Number of Rats Exam- ined	Total	Posi- tive	Nega- tive	Not Made	Total	Posi- tive	Nega- tive	Not Made
Albany.....	69	22*	11	10	1	47	5	40†	2
Other localities	47	0	0	0	0	47	0	44	3

* *Leptospiras* were demonstrated microscopically in the tissues of guinea-pigs which developed characteristic lesions following inoculation with material from 20 of these rats or on passage from these to other guinea-pigs. In the two instances in which guinea-pigs developed characteristic lesions but the *leptospiras* were not demonstrated in their tissues or on passage, the organisms had been seen in the rat kidney emulsions.

† *Leptospiras* were seen in cultures from the kidney emulsions of two rats, but failed to develop in transplants and their pathogenicity was not determined.

pig. Material from a number of rats was also inoculated into the medium recommended by Noguchi³⁷ for cultivation for the *leptospira*. For purposes of comparison and control, the rat urine and the kidney emulsions were, in most instances, examined for *leptospiras* under the dark field microscope.

The results of the microscopical examinations and animal inoculations are summarized in table 1. (The results of the cultural examinations are indicated only in the two instances in which they gave information not obtained by other methods.)

A *leptospira*, similar in pathogenic action in guinea-pigs to *L. icterohaemorrhagiae*, was found to be carried in the kidneys of 22 out of 69, or almost one third of the rats caught in Albany. No evidence of this infection was obtained, however, in the examination of 47 rats from

³⁷ J. Trop. Med. & Hyg., 1925, 28, p. 185.

other localities in the state. As Schüffner and Kuenen³⁸ and others have found the leptospira infection to be much more prevalent in adult than in young rats, the demonstration of the infection exclusively in the Albany rats may possibly be explained by the fact (noted at the time of these examinations) that the majority of the rats caught in Albany were larger than those captured elsewhere. No correlation was evident between the incidence of the leptospira infection in rats, and the occurrence of cases of the epidemic jaundice described, for the cases were less numerous in Albany than in any of the other localities from which rats were examined.

Darkfield examinations of the urine or kidney emulsions failed to detect leptospiras in 9 of the 22 rats in which they were demonstrated by guinea-pig inoculation. On the other hand, leptospiras were seen in kidney emulsions from 5 rats, and in cultures from kidney emulsions of 2 rats which produced no evidence of the infection when injected into guinea-pigs. While it is conceivable that the leptospiras in these 7 rats belonged to species other than *L. icterohaemorrhagiae*, their identity with, or at least their relationship to that organism was suggested by the results of later immunological tests in which the serums of 6 of these rats were found to agglutinate *L. icterohaemorrhagiae*. The failure to infect guinea-pigs in these instances, if not due to the attenuation of the organisms, may well be attributed to the insusceptibility of the animals inoculated, for marked variations in reaction were noted in the animals receiving virulent leptospiras, some developing fatal spirochaetosis, while others suffered a nonfatal infection or gave no indication of infection whatever.

Thus, exclusive of guinea-pigs in which the outcome was obscured by a bacterial infection, 34 received rat kidney emulsions containing virulent leptospiras, of which 21, or about two-thirds developed fatal spirochaetosis, and 13 survived. Among 10 surviving guinea-pigs, of which the temperatures were recorded daily, 8 had a marked, and 2 but a slight febrile reaction. In immunological tests, made 4 to 5 months after inoculation of these animals, agglutination was obtained with serums of five which had had high fever, and one of these was found at necropsy to have leptospiras in the kidneys. No agglutination was obtained with serum from one guinea-pig which had had fever, nor with serums from the two which had had but a slight rise in temperature nor from the three of which the temperature was not recorded.

Immunological Reactions of the Serums of Wild Rats.—Supplementary to the examinations for *L. icterohaemorrhagiae* described, immuno-

³⁸ Nederl. Tijdschr. v. Geneesk., 1923, 2, p. 2018 (Abstr., Brit. M. J., 1924, 1, p. 24).

logical tests were performed on the serums of a large number of the rats, with cultures of *L. icterohaemorrhagiae* isolated by Noguchi from wild rats in the United States³⁹ and in Central America.⁴⁰

To test the effect of rat serum on the leptospira in vitro, 0.05 cc. of serum was mixed with 0.05 cc. of a rich, actively motile culture of *L. icterohaemorrhagiae*, grown in rabbit serum medium, the mixture was diluted with 0.1 cc. of physiologic salt solution and after one-half hour at room temperature was examined under the darkfield microscope. A similar test was also made, in which the mixture of rat serum and culture was combined with 0.1 cc. of fresh guinea-pig complement, and the observation made after incubation for one-half hour at 37 C. This test was in all instances controlled by a test of the culture and guinea-pig complement without rat serum.

The effect of the rat serum on the leptospira in vivo was studied by the method of Pfeiffer, a mixture of 0.5 cc. serum and 0.5 cc. culture, diluted with 1.5 cc. salt solution, being injected into the peritoneal cavity of a guinea-pig, and portions of the fluid withdrawn after 15 minutes and 30 minutes for examination under the darkfield microscope. The protective properties of the rat serums could not be determined with the known strains of *L. icterohaemorrhagiae* then available, as they had become avirulent in consequence of continued cultivation in artificial medium.

In the examinations of serums from Albany rats (table 2), 35 out of 55, or almost two-thirds, gave a reaction with *L. icterohaemorrhagiae* in vitro, varying from slight agglutination to marked disintegration and lysis.* Reactions occurred with the serums of all the rats in this series in which leptospiras had been demonstrated microscopically, or by guinea-pig inoculation or culture, and with the serums from 11 of the 31 rats in which leptospiras had not been found. Pfeiffer tests, which were made on 34 of the serums, confirmed the reactions in vitro except in one instance, in which the serum from a rat infected with the leptospira failed to give the Pfeiffer reaction, but agglutinated slightly in vitro.

In the examinations of serums from rats outside Albany, no reaction was obtained with 32 which were tested in vitro only, nor with 10 which were tested in vitro and in vivo as well. These results, with serums of rats in which no evidence of the leptospira infection had been

* Although agglutination and immobilization were observed in vitro at 20 C., the effect was usually more marked at 37 C., so that the results given are those obtained at the latter temperature.

³⁹ J. Exper. Med., 1917, 25, p. 755.

⁴⁰ Ibid., 1919, 30, p. 95.

found, appeared, therefore, to control the significance of the reactions which were obtained with so large a proportion of serums from Albany rats.

Because of the discrepancies between the results of microscopical examinations and animal tests for leptospiras in Albany rats, the incidence of the infection determined by these methods (approximately 40%) is obviously an underestimation. While a serological reaction, even if specific, may not indicate existing infection, the observations of Schüffner and Kuenen³⁸ suggest that a rat once infected remains a carrier for life. It seems probable, therefore, that the actual frequency

TABLE 2
CORRELATION BETWEEN RESULTS OF EXAMINATIONS FOR LEPTOSPIRA ICTEROHAEMORRHAGIAE AND IMMUNOLOGIC TESTS OF SERUMS FROM ALBANY RATS

Results of Examinations for Leptospira icterohaemorrhagiae	Effect of Rat Serum on L. icterohaemorrhagiae in Vitro (at 37 C.)					Results of Pfeiffer Tests with L. icterohaemorrhagiae Com- pared with Reactions in Vitro			
	Num- ber Tested	Agglutination Immobilization, etc.			No Effect	Num- ber Tested	Pfeiffer's Reaction		
		Total	Marked	Slight			Obtained	Pfeiffer Test Negative	
								Agglu- tation	Slight and Agglu- tation
Typical spirochaetosis in guinea- pigs inoculated with rat urine or kidney emulsions.....	18	18	14	4	0	10	9	1	0
No evidence of spirochae- tosis in guinea-pigs inoculated with rat urine or kidney emulsions	6	6	4	2	0	3	3	0	0
Leptospiras seen in rat urine or kidney emul- sions or cultures from the latter..	31	11	10	1	20	21	9	0	12
Leptospiras not seen in rat urine or kidney emul- sions or in cul- tures.....	37	17	14	3	20	24	12	0	12
Total number of rats exam- ined.....	55	35	28	7	20	34	21	1	12

is more nearly indicated by the proportion of rats giving immunological reactions to *L. icterohaemorrhagiae* (about 60%). In view of the great prevalence of the infection thus indicated, a further systematic study of the leptospira isolated from Albany rats was undertaken to establish its relation to *L. icterohaemorrhagiae*.

Comparison of the Leptospira Isolated from Albany Rats with L. Icterohaemorrhagiae.—Morphology: The stained preparations were made by Miss Marion B. Coleman, who has made a special study of

the various methods of staining flagella. In shape, size, and manner of locomotion, and in its structure as seen under the darkfield microscope, the leptospira isolated from Albany rats conforms with descriptions of *L. icterohaemorrhagiae* published by Inada² in Japan and by Noguchi² in this country. Flagella, however, were demonstrated in stained preparations.

Martin, Pettit and Vaudremer,⁴¹ are the only authors to report staining the flagella of *L. icterohaemorrhagiae*. They used the methods of Loeffler⁴² and van Ermengem,⁴³ later the "Largine" method⁴⁴ of Ravaut and Ponselle⁴⁵ and the method of Casares-Gil. In their photographs of preparations impregnated with Largine, the leptospira appeared to have a fine threadlike appendage at each end, which terminated in a small spherical body.

In preparations of the Albany rat leptospira, stained by the method of Loeffler⁴² or by the modifications of that method recommended by van Ermengem,⁴³ Bunge⁴⁶ or Shunk,⁴⁷ no flagella were found. In specimens impregnated with Largine, however (fig. 1), the organisms were seen to have appendages such as Martin, Pettit and Vaudremer have described.

The method of staining with Largine, which follows in all essentials the procedure of Ravaut and Ponselle, is as follows:

Air dried films are fixed in a 1% solution of osmic acid for from two to five minutes, washed in running tap water and allowed to dry in the air. They are then immersed in a freshly prepared 2% solution of largine at 55 C. for two hours. The slides are then transferred, without preliminary washing, to a jar containing a 5% solution of pyrogallie acid, and after a few seconds are transferred to a second jar of the same solution and left for two minutes or more. They are then washed in tap water and dried in the air. These preparations have been found to remain in satisfactory condition for several weeks if kept in the dark, but they fade rapidly if exposed to the action of light or cedar oil.

A modification of the above method, in which largine is used after treatment of the film with Bunge's mordant, failed to show flagella, but gave clear definition of the spiral body (fig. 2).

Cultivation: The rabbit serum, semisolid medium of Noguchi was successfully employed for the isolation of the leptospira from infected animals, as well as for the maintenance of strains which had been under cultivation. Modifications of this medium, prepared with horse serum and bull serum respectively, which were used for the production of large amounts of culture, gave fairly good growth of the leptospira, but less luxuriant than that obtained in rabbit serum medium. The

⁴¹ Compt. rend. Soc. de Biol., 1916, 79, p. 1053.

⁴² Centralbl. f. Bakteriöl., I, O., 1889, 6, p. 209.

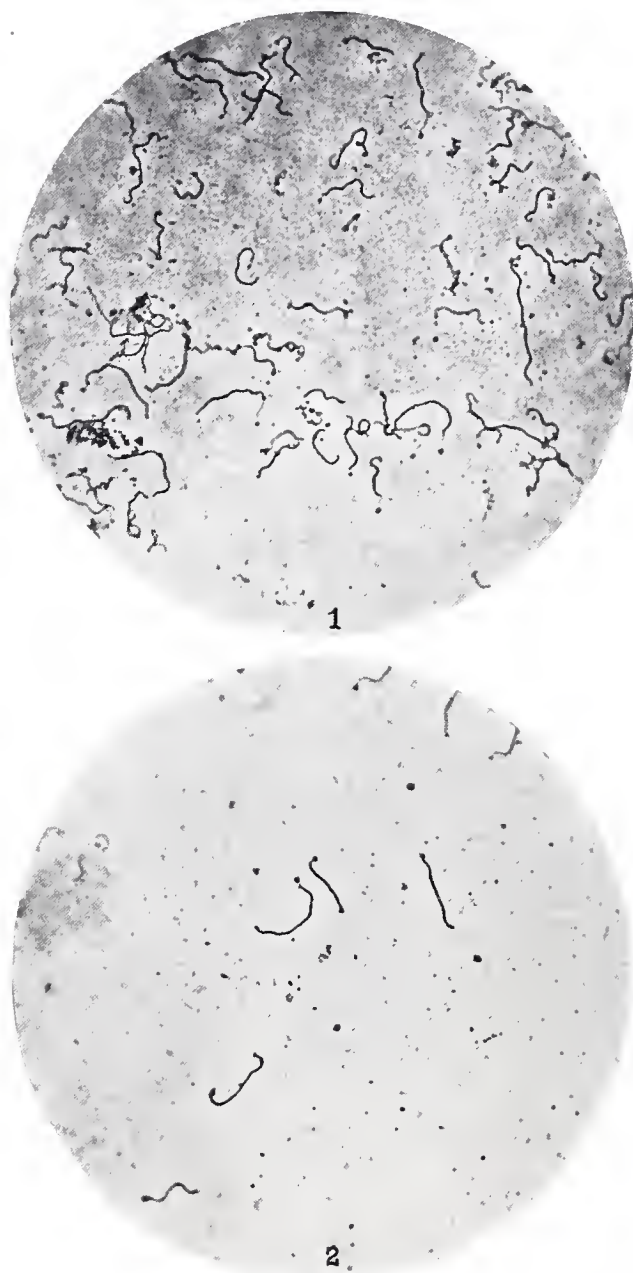
⁴³ Ann. Soc. de méd. de Gand., 1893, 72, p. 231.

⁴⁴ Martin, L.; Pettit, A., and Vaudremer, A.: Compt. rend. Soc. de Biol., 1917, 80, p. 197; Martin, L., and Pettit, A.: Monographie de l'Institut Pasteur, 1919.

⁴⁵ Compt. rend. Soc. de Biol., 1908, 2, p. 438.

⁴⁶ Fortschr. d. Med., 1894, 12, p. 462.

⁴⁷ J. Bact., 1920, 5, p. 181.



Figs. 1 and 2.—*Leptospira icterohaemorrhagiae* from culture in rabbit serum medium. Organisms impregnated with larginine (fig. 1) show appendages. Treatment with larginine after Bunge's mordant reveals spiral body (fig. 2).

leptospira was cultivated in this medium aerobically. Reducing the access of oxygen to the medium by covering the surface with oil, as originally recommended by the Japanese,⁴⁸ was tried with a few cultures but was not found to affect the rate of growth perceptibly.

With incubation at 26 C., fair growth of initial cultures from animals was obtained in from five to eight days, and growth of cultures adapted to artificial medium in from three to four days. For luxuriant growth, incubation for about ten days to two weeks was required. Although the longevity of the leptospira cultures at this temperature has not been exactly determined, active organisms have been found in cultures as old as 12 weeks. With incubation at 33 C., the rate of growth was found to be increased, but degeneration also appeared to be more rapid.

The viability, like that of *L. icterohaemorrhagiae*, is brief in culture medium contaminated with molds or bacteria, but typical lesions of spirochaetosis, and living leptospires have occasionally been found associated with intercurrent (or terminal) infections with bacteria, such as pneumococci, streptococci, or organisms of the paratyphoid group.

This leptospira, like the icterohaemorrhagic strains studied by Noguchi,² is destroyed within a few minutes by 10% solutions of bile salts and sodium oleate, but is not affected (observation within 5 hours) by a 10% solution of saponin.

Pathogenic Action. — Experimental Infection in Guinea-Pigs: Guinea-pigs experimentally infected with the Albany rat leptospira typically manifest the symptoms of fever, jaundice, albuminuria, etc., and at necropsy display the jaundice and extensive hemorrhages which Inada² and others have described in guinea-pigs infected with *L. icterohaemorrhagiae*.

Generalized, fatal infection with this leptospira has been induced in guinea-pigs by intraperitoneal, subcutaneous or intracutaneous injection, and by the application of infectious material to the shaved, slightly scarified skin of the abdomen. With organisms of high virulence, infection has also been induced in guinea-pigs by feeding, through a rubber tube, as well as by application of infectious material to the conjunctiva, or even to the apparently uninjured skin (the hair being clipped carefully with scissors but not shaved). The typical findings of

⁴⁸ Ito, T., and Matsuzaki, H.: J. Exper. Med., 1916, 23, p. 557.

jaundice and hemorrhage have been observed in guinea-pigs inoculated by all methods. In certain instances, however, animals inoculated on the mucous membranes have failed to show the severe hemorrhages in the subcutaneous and retroperitoneal tissues which are so characteristic in animals inoculated intraperitoneally, subcutaneously, or through the skin of the abdomen.

Since guinea-pigs are so readily infected with *L. icterohaemorrhagiae* through slight abrasions in the skin, inoculation on the lightly scarified skin of the abdomen has been successfully employed by Griffith⁴⁹ to separate that organism from contaminating bacteria. In one experiment in which this method of inoculation was tried with a liver emulsion containing the Albany rat leptospira in combination with paratyphoid bacilli (from a guinea-pig which had died of peritonitis) the animal used developed typical, uncomplicated spirochaetosis. In a second experiment, however, in which a mixture of leptospira and paratyphoid cultures was used, the guinea-pig developed a generalized infection with both organisms.

The incubation period in the case of guinea-pigs inoculated intraperitoneally with moderately large numbers of virulent leptospiras is ordinarily about 48 hours, the death or collapse of the animal occurring in five or six days. Following subcutaneous or intracutaneous injection of similar doses, the incubation period is slightly longer, and following inoculation by mouth, through the uninjured skin or mucous membrane, it is usually extended, so that death does not occur before the ninth or tenth day.

The virulence of the Albany rat leptospira, like that of *L. icterohaemorrhagiae*, is modified by animal passage as well as by cultivation in artificial medium. Guinea-pigs inoculated with material from rat carriers, as previously mentioned, frequently did not develop a fatal infection. In the case of those which were fatally infected, the duration of the disease varied from five to fifteen days and averaged about ten days. After passage through guinea-pigs, the virulence for these animals is increased, death occurring quite regularly in five or six days. The tendency to hemorrhage is more pronounced in guinea-pigs infected with such passage strains, but the jaundice is frequently less intense than in those inoculated with material from rats. When cultivated in artificial medium, on the other hand, with transfer at intervals of one or two weeks, a perceptible decrease in virulence has occurred in about two months, and a marked decrease in from four to six months.

Variations in Pathogenicity for Different Animals: Animals, other than guinea-pigs, which have been experimentally inoculated with the

⁴⁹ J. State Med., 1922, 30, p. 70.

rat leptospira have proved less susceptible. On account of the possibility of natural infections in other animals, particularly some of the domestic animals, the observations on their susceptibility to infection are important to record.

Full grown rabbits, after the inoculation of leptospira culture, have consistently shown but a transient febrile reaction, and even young rabbits (weight from 400 to 500 gm.) have but rarely shown symptoms of disease. The inoculation of young rabbits, either intravenously or intraperitoneally, with highly virulent passage strains, however, has induced a fatal infection. These animals have manifested the characteristic symptoms of fever, conjunctival congestion and jaundice, and their blood, taken before death, has produced typical spirochaetosis in guinea-pigs. Necropsy has revealed jaundice of the tissues, but hemorrhages have not been observed. The relative insusceptibility of rabbits to the leptospira has been noted by other investigators also. Uhlenhuth and Fromme⁵⁰ found that the virulence of the leptospira for these animals may be increased, however, for after passage of the organism through a series of young rabbits, not only jaundice, but hemorrhages were produced.

By the injection of extremely large doses, a fatal leptospira infection has been induced in young kittens. In one experiment, intraperitoneal inoculation of a kitten (weight about 400 gm.), with 10 cc. of rich, virulent culture, was followed by an infection fatal on the tenth day. The febrile reaction was slight, but the animal showed progressive emaciation and weakness, and on the eighth day was seen to have a hemorrhage in the iris of one eye. At necropsy jaundice of the tissues was observed, also hemorrhage on the lumen of the large intestine, and diploë. Since darkfield examination revealed only a few dead leptospiras in the urine and kidney emulsions, a mixture of the kidney and liver emulsions was inoculated into another kitten. This animal died in twelve days. Although this second kitten had shown no characteristic symptoms, and necropsy revealed no gross evidence of spirochaetosis, a guinea-pig inoculated with a mixture of the kidney and liver emulsions of this animal developed a typical, fatal infection and leptospiras were demonstrated in its tissues at necropsy. It may, therefore, be concluded that the leptospira was transmitted from the first to the second kitten and thence to the guinea-pig. No record has been noted in the literature of other workers having found the leptospira pathogenic for kittens.

White rats and white mice inoculated with the rat leptospira have commonly become carriers. No symptoms of disease have been noted in these animals, but when killed, five or six weeks after inoculation, they have been found to harbor in their kidneys leptospiras virulent for guinea-pigs and their serums have agglutinated leptospira cultures. Most of the published reports agree that these rodents rarely show symptoms of illness following inoculation with *L. icterohaemorrhagiae*. Inada,² found that 4 of 14 mice, and 1 of 2 white rats, succumbed with jaundice and hemorrhage. Uhlenhuth and Fromme⁵⁰ observed characteristic lesions in white rats following injection of massive doses. Haendel, Ungermann and Jaenisch⁵¹ also induced characteristic symptoms in mice, but only after increasing the virulence of the leptospira for these animals by the passage of peritoneal fluid from mouse to mouse.

⁵⁰ Ztschr. f. Immunitätsforsch. u. exper. Therap., 1919, 28, p. 1.

⁵¹ Arb. a. d. k. Gsndtsamte, 1918-19, 51, p. 42.

In studying the reaction of wild rats to inoculation with leptospira culture or passage strains, it was considered necessary to use animals, which, from serological tests, showed no evidence of previous leptospira infection. Two wild rats were selected, the sera of which did not agglutinate the rat leptospira. One was inoculated intraperitoneally with 1.0 cc. of virulent leptospira culture, the other with 4 cc. of liver emulsion from an infected guinea-pig. Neither animal showed symptoms of disease. When killed, two months later, their serums agglutinated leptospira culture; a leptospira was seen in the kidney emulsion of the rat inoculated with culture, but spirochaetosis did not develop in the guinea-pigs inoculated with the kidneys of either of the rats. Other investigators, including Griffith⁴⁹ and Nicolle and Lebailly⁵² have demonstrated virulent *L. icterohaemorrhagiae* in the kidneys of wild rats, at long intervals after inoculation. Similar results have been obtained by the authors also, but in these instances the possibility of previous, natural infection must be considered, for the rat serums, before inoculation, had given partial agglutination of the leptospira. No mention has been seen of other workers having employed serological tests to obtain evidence of leptospira infections in wild rats.

The pathogenicity of the rat leptospira for man was directly and conclusively proved when one of the authors developed an infection as a result of accidentally pricking her finger with the needle of a syringe containing a rich, recently isolated culture. As recorded in the preliminary report,¹ guinea-pigs inoculated with the blood of this patient died with the typical lesions of spirochaetosis, and their organs were found to contain morphologically typical, virulent leptospiras.

"Friday, February 3, while preparing for the inoculation of a rabbit with a virulent culture from a rat, one of the workers pricked her finger with the needle of the syringe containing the culture for inoculation. The rabbit was inoculated, and the worker cleaned and disinfected her wound. She had no symptoms of illness until the evening of Friday, February 10, when the febrile reaction started, together with general malaise, nausea and vomiting. The febrile reaction lasted ten days, the temperature rising to 104 on the third day, with remissions to 100 or 101, and persisted, rising to 103 and remitting, then gradually falling and reaching normal on the tenth day. Prostration was marked and increasingly so as the febrile reaction continued, but there was no material disturbance of the pulse rate-temperature ratio. The pulse rate fluctuated between 110 and 130 during the height of the febrile reaction. There were no signs of involvement of any of the organs, no bile or casts in the urine, and no significant albuminuria. There was no jaundice beyond a possible brief tinging of the sclerae. There was moderate leukocytosis at the beginning. In fact, the diagnosis was quite obscure until cultures of the leptospira were obtained."

Immunologic Reactions.—Action of Immune Serums on the Albany Rat Leptospira: The serums of wild rats naturally infected with the leptospira have been found to give agglutination in vitro, and agglutination and lysis in vivo, with cultures of the Albany rat leptospira, as well as with the Noguchi strains of *L. icterohaemorrhagiae*. Similar reactions have also been obtained with the serums of wild rats, white rats and mice which have become carriers following inoculation with

⁵² Compt. rend. Soc. de Biol., 1918, 81, p. 469.

the rat leptospira, and with the serums of guinea-pigs recovered from infection with that organism.

Monovalent immune rabbit serums, produced by repeated intravenous injection of rich, live leptospira culture, have produced marked agglutination even in high dilutions, and prompt destruction of the homologous organisms in vivo. Specific protective properties also have been demonstrated. In the case of one serum tested quantitatively, 0.001 cc. gave a guinea-pig partial protection, and 0.005 cc. complete

TABLE 3

PROTECTION TESTS AND PFEIFFER REACTIONS WITH LEPTOSPIRA ICTEROHAEMORRHAGIAE AND SERUM FROM PERSON INFECTED WITH ALBANY RAT LEPTOSPIRA, FEBRUARY, 1922

Serum	Date of Collection	Amount Serum, Cc.	L. icterohaemorrhagiae		Pfeiffer Reaction	Protection Tests with Inoculated Guinea-Pigs	
			Material	Amount, Cc.		Protection	Spirochetosis
Immune (Human)	5/12/22	0.5 to 0.1	Albany rat culture	0.5	+	Complete	0
		0.05	+	Death, 4 days no jaundice	?
		0.01.....	+	0	Fatal, 12 days
	2/ 1/26	0.005 to 0.0005.....	0	0	Fatal, 5 to 8 days
		1.0	Boston rat heavy emulsion of guinea-pig liver*.....	1.0	+	Complete	0
		0.1 to 0.1	(Dilute emulsion)*.....	1.0	—	Complete	0
Controls.....	2/ 1/26	0.01	(Dilute emulsion).....	1.0	—	0	Fatal, 9 days
		0.5	Albany rat culture	0.5	—	0	Fatal, 5 days
		1.0	Boston rat heavy emulsion guinea-pig liver.....	1.0	—	0	Fatal, 6 days
	(Salt solution)	1.0	(Dilute emulsion)*.....	1.0	—	0	Fatal, 5 days
		1.0	(Dilute emulsion)*.....	1.0	—	0	Fatal, 5 days
		1.0	(Dilute emulsion)*.....	1.0	—	0	Fatal, 5 days

0 = negative result, and — = no test made.

* The liver of a small guinea-pig (200 Gm.) which died of leptospira infection was ground and suspended in 15 cc. of salt solution. In the controls this emulsion was diluted to contain 1 leptospira in every 10 to 15 microscopic fields.

protection against 0.5 cc. of leptospira culture, whereas the control animal which received the same dose of culture plus 0.5 cc. of normal rabbit serum died of spirochaetosis in five days.

In the instance of accidental human infection, also, the specific agglutinative, lytic and protective properties of the serum were demonstrated after recovery. As shown in table 3, a specimen of serum taken three months from the onset gave a guinea-pig complete protection in 0.1 cc. against a virulent culture of the Albany rat leptospira. The protective property was remarkably persistent in this case, for a specimen of the

serum taken four years from the onset gave complete protection in 0.1 cc. against another virulent rat strain of *L. icterohaemorrhagiae*.*

The complement fixation test performed with antigen prepared from cultures of the leptospira by an adaptation of the absorption technic used in preparing tubercle antigens⁵³ has appeared to be inferior to the Pfeiffer, agglutination or protection tests in the demonstration of immune substances. The specific fixation obtained with the serum of a human being and of animals known to have been infected with the rat leptospira was only slight, and the degree of cross-fixation obtained with certain syphilitic serums was marked.

Cross-Immunity Reactions Between the Albany Rat Leptospira and Other Strains of *L. Icterohaemorrhagiae*: Cross-agglutination has been demonstrated between the strains of the Albany rat leptospira and the Noguchi strains of *L. icterohaemorrhagiae* group 8 and American No. 2, although the degree of agglutination could not be accurately compared because of the variation in the motility of the cultures.

Pfeiffer reactions have been obtained with the Albany rat strains and the serums of rabbits immunized to the group 8 and American No. 2 strains of *L. icterohaemorrhagiae*; they have also been obtained with the group 8 and American No. 2 cultures, and the serums of rabbits immunized to strains of the Albany rat leptospira. Moreover, the serum of a rabbit immunized to the American No. 2 strain, which is avirulent, has been shown to give complete protection against a virulent culture of the Albany rat strain. These cross-immunity reactions indicate that the Albany rat leptospira is immunologically identical with the group 8 and American No. 2 strains of *L. icterohaemorrhagiae*, which from Noguchi's studies have been found identical with the European and Japanese strains of human origin.

Cross-Immunity Reactions Between the Albany Rat Leptospira and *L. Icteroides*: By protection tests, a close immunological relationship has been demonstrated between the Albany rat strain of *L. icterohaemorrhagiae* and Noguchi's strain of *L. icteroides*, Palmeiras No. 3,⁵⁴ (table 4). Guinea-pigs were completely protected against infection with a highly virulent passage strain of this organism by 1.0 cc. of serum taken four years after recovery from the case of accidental human infection with the Albany rat leptospira. With 0.1 cc. of serum from this

* The second test was kindly made by Dr. A. W. Sellards, with a strain of *L. icterohaemorrhagiae* isolated by him from a wild rat in Boston.

⁵³ Wadsworth, A.; Maltaner, F., and Maltaner, E.: *J. Immunol.*, 1925, 10, p. 241.

⁵⁴ Monograph of the Rockefeller Inst. for M. Research, 1924, No. 20.

case, one guinea-pig was completely protected against icteroides infection and another, although it showed a slight conjunctival congestion and tingeing of the sclera a few days after inoculation, shortly recovered and remained alive and well. One-tenth cc. was, likewise, the least amount of serum from this patient which had been found to give complete protection against infection with the homologous strain of *L. icterohaemorrhagiae*.

Likewise, in a case of spirochaetosis icterohaemorrhagiae described by Manson-Bahr,³⁰ the patient's serum, after recovery, protected against both *L. icteroides* and *L. icterohaemorrhagiae*. It is not evident that this serum was as highly protective against the yellow fever strain as against *L. icterohaemorrhagiae*, however, for the *L. icteroides* culture

TABLE 4

ACTION ON *LEPTOSPIRA ICTEROIDES* (STRAIN PALMEIRAS NO. 3) OF SERUM FROM PERSON INFECTED, FEBRUARY, 1922, WITH THE ALBANY RAT *LEPTOSPIRA*

Serum		L. icteroides (Guinea-Pig Liver Emulsion) Cc.	Guinea-Pigs Inoculated	
Kind	Amount, Cc.		Number	Result
Icterohaemorrhagiae; immune, human; collected 8/9/26.....	1.0	0.5	3	All completely protected
	0.1	0.5	2	1 completely protected; 1 transient fever and jaundice with recovery
Normal human, 1.....	1.0	0.5	2	Both typical icteroides infection, fatal in 5 days
Normal human, 2.....	1.0	0.5	2	Both typical icteroides infection, fatal in 4 days
(Salt solution)	1.0	0.5	2	Both typical icteroides infection, fatal in 4 and 6 days respectively

employed was one stated to be of low virulence, and the minimum amount of the patient's serum required for protection against each strain was not determined. The marked, and almost equal protection against both yellow fever and rat strains of leptospiaras obtained with the serum of the case of icterohaemorrhagiae infection studied by the authors, is especially remarkable since a similar effect of yellow fever convalescent serum upon rat strains of *L. icterohaemorrhagiae* has not been obtained by Noguchi; in fact, the serum of the patient from whom the Palmeiras No. 3 strain of *L. icteroides* was isolated failed to affect *L. icterohaemorrhagiae*. The serums of persons recovered from yellow fever, studied by Noguchi,⁵⁵ in most instances, gave the reaction of Pfeiffer with *L. icteroides*, but never with *L. icterohaemorrhagiae*. Moreover, the reaction of such serums on *L. icteroides* was in some

⁵⁵ Am. J. Trop. Med., 1924, 4, p. 131.

cases only partial so that a guinea-pig was not protected against an ultimate fatal *icteroides* infection. So far as may be judged from animal experiments, therefore, the immunity to *L. icteroides* that develops during an attack of yellow fever may actually be less than the immunity to that organism which has been demonstrated in this instance four years after recovery from infection with the rat leptospira.

SUMMARY

From a review of the literature it is evident that leptospiral infection, which is designated by the Japanese as *spirochaetosis icterohaemorrhagica*, is distinct from other forms of so-called infectious or epidemic jaundice. Despite the variations which occur in individual instances, the clinical manifestations in groups of cases mark *spirochaetosis* as an acute infection with severe toxic effect upon the entire organism, characteristically indicated by complete and sudden prostration, a marked febrile reaction, intense muscular pains, hemorrhage, jaundice and nephritis. Epidemiologically, also, *spirochaetosis* is unique; it occurs sporadically, or in localized groups of cases, almost exclusively in persons living in an insanitary environment where wild rats, which are carriers of the infectious agent, are apt to be numerous.

The investigation of the jaundice outbreak in New York State failed to reveal the presence of *L. icterohaemorrhagiae* in the blood or urine of persons affected, or any immunological reactions to this organism with the serums of those recovering from the disease. Moreover, the clinical and epidemiologic features of this outbreak differed essentially from those of *spirochaetosis*. Its etiology, therefore, remains obscure.

Supplementary to the study of human cases of jaundice, an extensive survey was made to determine the presence of *L. icterohaemorrhagiae* in wild rats. A leptospira was demonstrated in the kidneys of over 40%, and immunologic tests of the rat serums indicated its presence in over 60% of the rats captured in Albany.

The leptospira isolated from Albany rats is apparently identical in morphology, cultural reactions, pathogenic properties, and immunological reactions with *L. icterohaemorrhagiae*.

The pathogenicity of the rat leptospira for human beings, which had been assumed from the identity of the leptospira strains from rats and human cases, was directly demonstrated by a case of accidental human infection with the Albany rat leptospira.

A study of the immunologic reaction in this case indicated a close relationship of the rat leptospira to a strain of *L. icteroides* isolated by Noguchi from a case of yellow fever. Even four years after recovery, the serum of this patient gave marked protection to guinea-pigs against a highly virulent passage strain of *L. icteroides* as well as against *L. icterohaemorrhagiae*. The serums of yellow fever convalescents studied by Noguchi, on the other hand, not only have failed to show a similar effect upon *L. icterohaemorrhagiae*, but have failed in some instances to protect guinea-pigs against yellow fever strains of *icteroides*.

BRILLIANT GREEN AND ITS USE IN AN ENRICHMENT MEDIUM IN THE ISOLATION OF TYPHOID AND PARATYPHOID ORGANISMS

MORRIS L. RAKIETEN AND LEO F. RETTGER

From the Laboratory of General Bacteriology, Yale University, New Haven, Conn.

It has long been known that certain dyes, particularly those of the triphenyl methane and flavine series, have a bacteriostatic, and in some instances, strong germicidal action. This deportment of the dyes varies with the dyes and with the bacteria subjected to them. Advantage has been taken of this fact by numerous investigators in their attempts to devise selective and differential mediums and methods for the isolation and identification of species and types, particularly those of the colontyphoid-paratyphoid group.

Among the dyes which have perhaps received most attention in recent years are gentian violet, brilliant green and malachite green. Since the work of Loeffler¹ and Conradi² on the use of malachite green in the isolation of the typhoid bacillus, and of Browning, Gilmour and Mackie³ on the use of brilliant green in an enrichment medium for the same purpose, numerous methods have been advocated as substitutes or modifications of these.

A study of various methods which were advocated led the authors to conclude that a combination of a reliable enrichment medium and efficient plating method should offer much toward the successful isolation of the typhoid and paratyphoid bacilli from the feces of patients and carriers.

A modification of the method of Robinson and Rettger⁴ in which the principle of the Browning, Gilmour and Mackie brilliant green enrichment medium was followed, and in which a slightly modified Endo⁵ medium was employed, was decided upon for the present investigation. This combination of mediums and methods gave quite consistent results in the present investigation when the principles

Received for publication, March 24, 1927.

¹ Deutsch. med. Wehnschr., 1906, 32, p. 1330.

² Centralbl. f. Bakteriöl., Ref. 1, 1908, 47, p. 47.

³ J. Hyg., 1913, 13, p. 335.

⁴ J. M. Res., 1916, 34, p. 363.

⁵ Centralbl. f. Bakteriöl., I. O., 1904, 35, p. 501.

involved were rigidly adhered to, even when others failed. Furthermore, the whole process is simple and requires very little time.

The writers realized from the beginning that the brand of dye used, the concentration of the dye, and the H-ion concentration of the enrichment medium must be given serious consideration.

Initial Experiments.—The methods of Browning, Gilmour and Mackie³ and of Robinson and Rettger⁴ were employed in preliminary experiments in which three commercial brands of brilliant green were used. The enrichment medium was 2% peptone water containing 0.5% sodium chloride, and definite amounts of brilliant green. The reaction was P_H 6.8-7.0.

The dilutions of the dye ranged from 1:250,000 to 1:1,000,000, and were prepared from 1% stock solution as follows. Immediately before use a 1:10,000 dilution was made by adding 0.1 cc. of the stock solution to 9.9 cc. distilled water. From this solution, varying but definite amounts were added to five test tubes containing 5 cc. of the peptone water to give the following dye dilutions: 1:125,000, 1:165,000, 1:250,000, 1:500,000 and 1:1,000,000. To each of these tubes were added 4 mm. loopfuls of diluted normal feces which had been inoculated with an 18 hour culture of *Bact. typhosum*. (Hopkins and Rawlins strain). After incubation of the tubes for 24 hours at 37 C, Endo plates were streaked with a loopful of the contents, and incubated 18 to 24 hours at 30 C. Russell's medium was then inoculated with typhoid-like colonies, and further confirmatory tests made.

The results were unsatisfactory in that in almost all of the tubes the brilliant green was reduced or precipitated and its selective action thereby lost. This, it soon became apparent, was due to distinct alkalination of the tubes. Robinson and Rettger⁵ had observed this same phenomenon, but only in their highest dilution of the dye, 1:300,000. In the few tubes in which the dye was retained in solution (1:165,000) the growth was as a rule chiefly that of *B. coli*, as was indicated on the Endo plates.

With the cooperation of the junior author, Greenberg⁶ in the summer of 1922 made a study of the relation of H-ion concentration to the efficacy of brilliant green as a bacteriostatic agent in peptone water, and found that the P_H of the medium plays a very important part in the action of this dye, and that to hold the dye in solution the H-ion concentration must not be permitted to go above P_H 7.0. This important observation was further substantiated by the present authors, and by Stearn and Stearn.⁷

A number of experiments were conducted with *Bact. coli*, *Bact. aerogenes* and five different strains of *Bact. typhosum* in pure culture, and in fecal suspensions inoculated with these organisms. Tubes of 2% peptone water containing the brilliant green in dilutions of 1:50,000 to 1:1,000,000, and having reactions of P_H 6.0, 6.4 and 6.7 were used as enrichment tubes. In this, as in all later experiments, the modified Endo medium of Robinson and Rettger was used for plate isolation. The results are briefly summarized as follows:

⁶ Unpublished data.

⁷ J. Bact., 1926, 11, p. 345.

In the tubes having a reaction of P_H 6.0 the brilliant green remained in solution and was effective against *Bact. coli* in a dilution of 1:500,000. The H-ion concentration remained on the acid side of P_H 7.0. A distinct inhibitive action against *Bact. typhosum* was exerted in dilutions of 50,000 to 100,000. Optimum concentration for selective action on this organism ranged from 1:165,000 to 1:500,000. While a dilution of 1:1,000,000 favored the typhoid bacillus still further, there was such a slight restraining action on *Bact. coli*, and especially in the presence of organic matter, that this concentration was ineffective for practical isolation purposes. In all of the higher dilutions the dye had little if any restraining action on *Bact. typhosum*.

In tubes having a P_H of 6.4 the peptone in most instances was rendered alkaline in the presence of fecal material, and the dye was reduced or precipitated in dilutions of 1:500,000 and 1:1,000,000, when the typhoid bacillus was used, and in lower dilutions in the presence of *Bact. coli* and *Bact. aerogenes*.

In tubes having an initial P_H of 6.7 the brilliant green was decolorized or reduced in the dilutions which should be optimum for the preferential growth of *Bact. typhosum*.

These experiments demonstrated conclusively that brilliant green exerts a marked restraining action on *B. coli* so long as the medium remains acid, but that as soon as the peptone water becomes alkaline (P_H 7.0.+) the dye is rendered more or less ineffective in its higher dilutions, with or without the presence of feces or other added organic matter. The addition of fecal material hastens this change in reaction, chiefly owing to the large numbers of bacteria it conveys; that is, to increased bacterial metabolic activities, and partly to the added organic matter as such. Even an initial reaction as low as P_H 6.0 does not always insure against alkalization with decrease or complete loss of dye activity.

Of the three American brands of brilliant green employed in the above experiments, one (L) exerted a much more pronounced inhibitive action on the typhoid bacillus than the other two, and it soon became apparent that the choice of brilliant green is extremely important. It should be said, however, that at least fairly uniform results were obtained with the different samples of Grüber's brilliant green which were used.

USE OF BUFFER MIXTURES IN THE BRILLIANT GREEN ENRICHMENT MEDIUM

The necessity of controlling the reaction of the medium so as to preserve the full action of the dye was strongly impressed on the writers almost from the beginning. A solution of the problem was sought in

the employment of chemical buffering agents in the proper proportions to stabilize the medium within the desired P_H range and yet exert no marked bacteriostatic action on the typhoid and paratyphoid organisms.

The optimum H-ion concentration for *Bact. typhosum* was shown by Shohl and Janney,⁸ Fellen,⁹ Schoenholz and Meyer¹⁰ and Cluzett, Rochaix and Kofman¹¹ to be between P_H 6.0 and 7.0. Furthermore, it was shown by Greenberg, and by ourselves that it is within this range that the brilliant green is most effective.

After some experimenting the following two phosphate buffer mixtures were employed.

<i>Buffer Solution 1</i>		<i>Buffer Solution 2</i>	
K_2HPO_4	8.5 gm.	K_2HPO_4	7.5 gm.
KH_2PO_4	6.5 gm.	KH_2PO_4	7.5 gm.
Water	155 cc.	Water	155 cc.
Reaction = P_H 6.8		Reaction = P_H 6.5	

These two solutions constituted the stock buffer solutions for use in the brilliant green peptone water. Varying amounts were added to the tubes containing the medium and heavy fecal suspensions in order to determine the minimum amount of buffers required to keep the P_H of the medium within the limits of optimum growth of *Bact. typhosum*, and of dye action.

A 2% solution of peptone water having a reaction of P_H 6.8 was tubed in 5 cc. amounts, and to each of the tubes were added varying amounts of buffer solution 1 (10% phosphate, P_H 6.8). One series of the sterilized tubes was inoculated with one loopful of an 18 hour culture of *Bact. typhosum* 3, and a second was treated with a loopful of heavy suspension of feces, inoculated with typhoid bacilli. Both sets of tubes were incubated for 24 hours at 37 C, and plated on Endo medium by the usual streaking method. H-ion determinations were made at the same time on all of the tubes, by the colorimetric method. The results are given in table 1.

The results show that 0.4 cc. of the buffer solution 1, or approximately 1%, was sufficient to maintain the initial reaction, P_H 6.8, in the inoculated tubes not containing the feces, for a period of 24 hours, and that this concentration of the buffer has apparently no restraining effect on the growth of the organism. However, in the tubes containing the fecal suspension this amount of phosphate solution in many instances was not sufficient to hold the P_H below 7.0, though it did not permit it to go appreciably above.

⁸ J. Urol., 1917, 1, p. 211.

⁹ J. Infect. Dis., 1919, 25, p. 444.

¹⁰ Ibid., 1921, 28, p. 384.

¹¹ Compt. rend. Soc. de. biol., 1924, 178, p. 1638.

These experiments were repeated, but with a medium having an initial reaction of P_H 6.5, and treated with different amounts of buffer solution 2 (10% phosphate, P_H 6.5), in order to allow a wider zone between this and 7.0+. The data are summarized in table I.

From these results it is quite apparent that 1% of the 10% phosphate buffer solution 2 is sufficient to keep the inoculated peptone water, even when treated with one loopful of fecal suspension, well within P_H 7.0.

Several experiments were conducted with buffer mixture 2 (P_H 6.5) in which *Bact. coli*, *Bact. aerogenes*, *Bact. alkaligenes* and *Proteus vulgaris* were used, in place of the typhoid bacillus. In no instance did the H-ion concentration

TABLE 1
THE INFLUENCE OF DIFFERENT AMOUNTS OF THE PHOSPHATE BUFFER ON GROWTH OF BACT. TYPHOSUM AND ON H-ION CONCENTRATION

Cc. of Buffer (10%) in 5 Cc. Peptone Water	Medium Having Initial Reaction, P_H 6.8				Medium Having Initial Reaction, P_H 6.5			
	B. typhosum 3		Fecal Emulsion		Bact. typhosum		Fecal Emulsion	
	Resulting		Resulting		Resulting		Resulting	
	Growth	P_H	Growth	P_H	Growth	P_H	Growth	P_H
1.0	±	6.8	±	6.8	±	6.5	±	6.5
.9	±	6.8	±	6.8	±	6.5	±	6.5
.8	+	6.8	+	6.8	+	6.5	+	6.5
.7	+	6.8	+	6.8	+	6.5	+	6.5
.6	+	6.8	+	6.8	+	6.5	+	6.5
.5	+	6.8	+	6.9	+	6.5	+	6.5
.4	+	6.8	+	6.9	+	6.5	+	6.5
.3	+	7.0	+	7.2	+	6.8	+	6.8
.2	+	7.2	+	7.4	+	7.0	+	7.1
.1	+	7.5	+	7.4	+	7.2	+	7.4

+ = growth positive, like control, and ± = growth slightly restrained.

exceed P_H 7.0. In other words, 0.4 cc. (1%) of buffer solution 2 added to peptone water of the same P_H 6.5, was sufficient to prevent alkalization by any one of these organisms, under the conditions of the test.

From the foregoing results it appeared to the writers that 1% mixed phosphate buffer 2 (P_H 6.5) of the composition shown, contained in a 2% peptone water of the same P_H should offer a solution of the altogether too frequent failures that are obtained with the Browning, Gilmour and Mackie brilliant green enrichment method.

Success will depend, therefore, on two important factors related to the dye itself; first, the proper amounts of the dye to restrain *B. coli* (and other ordinary fecal organisms) and allow the typhoid bacillus to grow, and second, composition of the medium which will enable the

brilliant green to remain in solution, and exert its full action. The latter condition is made possible by a suitable chemical buffering agent.

A BRIEF COMPARATIVE STUDY OF THE EFFICACY OF SIX
DIFFERENT SAMPLES OF BRILLIANT GREEN

Besides two American brands carried in the laboratory stock, four other domestic brands furnished by Dr. Conn (labelled NBG1, EBg1, PDR, and C + B2) were used.

The enrichment medium was the 2% peptone water buffered at P_H 6.5 with phosphate solution. The concentrations of the dye were 1:100,000, 1:125,000, 1:165,000, 1:250,000, and 1:500,000. Both gram-positive and gram-negative organisms served as test bacteria. In one set of experiments with these organisms, 4 mm. loopfuls of 18 hour broth culture were used with and, in another, without, heavy fecal suspension. The tubes were incubated at 37 C. for 24 hours, when Endo plates were streaked and incubated at 34 C for 20 to 24 hours.

The above six samples of dye were compared with each other and brilliant green which had been thoroughly tried out, as "standard." Plain buffered peptone water tubes inoculated with the same organisms were used as controls.

The results obtained with a representative sample of Gröbler's brilliant green are shown in table 2.

The results show that the different strains of *Bact. typhosum* were resistant to the dye in concentrations of 1:125,000 to 1:500,000, and that the paratyphoid organisms were even more resistant than the typhoid. *Bact. coli* was, however, markedly arrested in these same concentrations, though *Bact. aerogenes* appeared on the whole to be quite indifferent.

It will be noted, also, that the dye was decidedly inhibitive towards the gram-positive organisms; also against the two strains of *Proteus vulgaris* employed, and against *Bact. abortus* and *V. cholerae*. Finally, *Bact. alkaligenes* showed considerable tolerance for the dye and the colonies on the Endo medium were quite similar to those of *Bact. typhosum*. This observation is of much significance in showing that *Bact. alkaligenes* colonies may easily be mistaken for typhoid. In fact, such instances have occurred in the present study.

A sample of C + B (old) brilliant green kept in the laboratory stock collection for some time was tested out on the same organisms and in the same way as the Gröbler samples. The results of this experiment were similar to those obtained with the Gröbler samples (table 2). This sample was indeed as satisfactory as the imported product. It has been used frequently by us in the isolation of *Bact. typhosum* from artificially infected feces, with considerable success.

The other sample of dye, however, labeled C + B2, and obtained from Dr. Conn, had a marked inhibitory effect on *Bact. typhosum* and the paratyphoid organisms in the higher dilutions. In fact, this sample had a restrain-

ing action on practically all of the organisms studied. Because of its bacteriostatic action on the typhoid and paratyphoid bacilli in the dilutions in which the standard samples were not inhibitive, its further use was not to be considered.

Results more or less similar to those of the last experiment were obtained with the domestic brand EBg1, though the restraining action toward the typhoid strains was on the whole much less pronounced.

TABLE 2
THE INFLUENCE OF GRÜBLER'S BRILLIANT GREEN ON VARIOUS ORGANISMS

Bacteria Tested	Organisms Alone (Dye Dilutions)					Organisms Plus Fecal Suspensions (Dye Dilutions)				
	1:100,000	1:125,000	1:165,000	1:250,000	1:500,000	1:100,000	1:125,000	1:165,000	1:250,000	1:500,000
<i>Staphylococcus aureus</i>	0	0	0	0	0	0	0	0	0	0
<i>Streptococcus</i>	0	0	0	0	0	0	0	0	0	*
<i>B. proteus</i> (N. Y. II).....	0	0	0	0	*	0	0	0	*	x
<i>B. proteus</i> (Mirabilis).....	0	0	0	0	*	0	0	0	*	x
<i>B. dysenteriae</i> (Flexner).....	0	0	0	*	*	0	0	*	*	±
<i>B. pyocyaneus</i>	±	±	±	+	+	±	±	±	±	±
<i>B. aerogenes</i> (Soil 3).....	±	±	±	+	D	+	+	+	D	D
<i>B. aerogenes</i> (Soil 1).....	+	+	+	+	+	+	+	+	+	+
<i>B. aerogenes</i> (feces).....	+	+	+	+	+	+	+	+	+	D
<i>V. cholerae</i>	0	0	0	0	0	0	0	0	0	0
<i>B. pullorum</i> (G 24).....	x	x	+	+	+	x	x	+	+	+
<i>B. pullorum</i> (R 16).....	±	±	+	+	+	+	+	+	+	+
<i>B. abortus</i> (Bang).....	0	0	0	0	0	0	0	0	0	0
<i>B. typhosus</i> (S).....	±	x	±	+	+	x	x	+	+	+
<i>B. typhosus</i> (R).....	0	x	+	+	+	*	x	+	+	+
<i>B. typhosus</i> (19).....	*	*	±	+	+	*	±	+	+	+
<i>B. typhosus</i> (30).....	*	±	+	+	+	x	±	+	+	+
<i>B. typhosus</i> (L. H.).....	0	x	±	+	+	*	±	+	+	+
<i>B. typhosus</i> (C).....	*	x	+	+	+	*	±	+	+	+
<i>B. alcaligenes</i>	0	*	x	±	±	0	x	±	+	+
<i>B. paratyphosus</i> A.....	±	+	+	+	+	+	+	+	+	+
<i>B. paratyphosus</i> B.....	+	+	+	+	+	+	+	+	+	+
<i>B. coli</i> (H 446).....	*	*	0	0	0	x	*	*	0	0
<i>B. coli</i> (R. A. K.).....	*	*	0	0	0	*	x	0	0	0

+ = growth as in control, ± = slight restraint, x = marked restraint, 0 = no growth, * = only a small number of colonies on Endo plates, and D = dye decolorized; not plated.

Sample of dye PDR was also very unsatisfactory, but differed from the last two in that it had more marked inhibitory influence on *Bact. typhosum* than on *Bact. coli*, though this condition may have been due, in part at least, to the presence of *Bact. aerogenes* in the feces employed and a heavy growth in the enrichment medium. Considerable reduction of the dye occurred, without alkalization.

The last of the series of samples, NBG1, exerted a marked bacteriostatic action toward both the typhoid and the colon bacillus, and no appreciable selective action could be observed. This brand was regarded as unsatisfactory, also.

A satisfactory brand of dye for use in the enrichment medium must hold in check the growth of *Bact. coli* in the medium and at the same time have very little or no inhibitory action on the typhoid and paratyphoid organisms in dye concentrations of 1:125,000 to 1:500,000. The different samples of Grüber and one of the American brands (C + B1) possessed this property. Because of the apparently uniform and satisfactory properties of the Grüber samples they were used as the standard brand in this investigation. The other domestic brands were unsatisfactory, and had to be rejected, particularly because of their decided restraining action on *Bact. typhosum* and the paratyphoids.

A significant feature of all the brands of brilliant green employed here is the absence of any restraining action on the strains of *Bact. aerogenes* used. This observation is not in accord with those of Krumwiede and Pratt¹² who found that this dye in dilutions of 1:100,000 to 1:300,000 exerts a retarding influence on this organism. *Bact. aerogenes* is met with occasionally in feces, and when present, it, or at least some strains of this organism, will render the problem of typhoid and paratyphoid isolation for the time difficult.

McConkey¹³ in a study of 497 strains of organisms isolated from 76 samples of various origin, claimed that *Bact. aerogenes* is rarely found in human and animal feces (6.3% of the samples tried). This is quite in agreement with our own observations. Furthermore, Clark and Lubs¹⁴ tested 113 organisms from 17 human subjects and found *Bact. aerogenes* to be present in only six instances, or 5.6% of the strains isolated. Levine¹⁵ made a study of lactose fermenters obtained from the feces of the horse (19), pig (21), cow (20), sheep (22), and man (25), and failed to find a single methyl red negative organism among the 107 examined. These results were further confirmed by Wood.¹⁶

INFLUENCE OF BRILLIANT GREEN ON THE MOTILITY OF *BACT. TYPHOSUM*

For the successful isolation of the typhoid and paratyphoid organisms definite optimum concentrations of the dye are necessary. These concentrations lie between 1:125,000 and 1:500,000, and vary more or less with the different strains.

Bact. typhosum is under normal conditions actively motile, whereas *Bact. coli* is at best but sluggishly motile. Concentrations of brilliant green in the enrichment medium in dilutions lower than 1:125,000 were found to affect the movements of the typhoid bacillus. The great majority of rods exhibited Brownian movement only, while others

¹² J. Exper. Med., 1914, 19, p. 501.

¹³ J. Hyg., 1909, 8, p. 322.

¹⁴ J. Biol. Chem., 1916, 25, p. 479.

¹⁵ J. Bact., 1918, 3, p. 253.

¹⁶ J. Hyg., 1919, 20, p. 60.

became massed in irregular groups or clumps. The picture was not at all unlike what is seen in ordinary typhoid agglutination with a specific serum.

The study of the influence of the dye on motility was made on the various strains employed in this entire investigation. They were transferred every day for four days in a beef broth medium having a reaction of P_H 6.5, and incubated at 37 C. for 20 hours. At the end of the last incubation period hanging drop preparations were made and examined. In every instance the organisms were actively motile.

TABLE 3
THE INFLUENCE OF BRILLIANT GREEN (GRÜBLER AND C&B1) ON THE MOTILITY AND GROWTH OF BACT. TYPHOSUM

Brilliant Green	Strains Tested	1:100,000		1:125,000		1:165,000		1:250,000 and 1:500,000	
		Microscopic Examination	Growth (Plate)	Microscopic Examination	Growth (Plate)	Microscopic Examination	Growth (Plate)	Microscopic Examination	Growth (Plate)
Grübler's	Rawlins	BA	*	BM	x	M	±	M	+
	C.	BA	*	BA	*	M	±	M	+
	3	0	0	BM	x	M	±	M	+
	19	B	*	BM	x	M	±	M	+
	S.	BM	*	BM	x	M	+	M	+
	M.	0	0	M	x	M	+	M	+
	L. H.	BA	0	BM	*	M	+	M	+
C + B 1	R.	B	*	BM	x	M	+	M	+
	Rawlins	0	0	BA	*	BM	±	M	+
	C.	0	0	BA	*	BM	±	M	+
	3	0	0	BM	*	M	+	M	+
	19	B	0	BM	x	BM	±	M	+
	S.	B	*	B	*	M	+	M	+
	M.	0	0	0	0	BM	±	M	+
	L. H.	B	*	BM	x	M	+	M	+
	R.	0	0	BM	x	M	+	M	+

BA = Brownian movement, also clumping; B = Brownian movement, no clumping observed; BM = motility when observed, sluggish; and M = real motility. + = growth on plate like control, ± = growth slightly restrained, x = growth markedly restrained, * = several colonies, 0 = no organisms observed; no growth on plate.

One % stock solutions of Grübler and C + B1 samples of brilliant green were made in distilled water, and from these a 1:10,000 dilution for immediate use. One-tenth cc. of an actively motile culture of Bact. typhosum was added to tubes containing 5 cc. buffered peptone water (P_H 6.5), and concentrations of brilliant green of 1:100,000, 1:125,000, 1:165,000, 1:250,000, and 1:500,000. The tubes were incubated for 20 hours at 37 C. Hanging drop examinations were then made, and at the same time a loopful of the culture material from each tube was streaked on Endo medium, the last step as a check on the microscopic examinations. At least 20 fields were examined in every depression slide mount.

In concentrations of from 1:165,000 to 1:500,000 of the dye no restraining action on motility was observed; nor on the growth of *Bact. typhosum*, or at most but very little. A slight selective, and at the same time inhibitive, action was exerted on the organism in 1:125,000 dilution, and with the exception of one strain (M), the dye in this concentration caused the organisms to be sluggish. In culture M all of the bacilli appeared actively motile. In concentrations of 1:100,000 a tendency of the organisms to form clumps was very evident, and Brownian movement alone was observed. All of the fields of strain L.H. showed clumped organisms, and the plates failed to develop any colonies.

There is a marked similarity in the concentrations 1:125,000 and 1:500,000 between the action of the C & B1 and the Grüber dye. There is a real difference, however, in the 1:165,000 concentration, especially in its effect on the motility of *Bact. typhosum* Rawlins, C19 and M. In each instance the majority of the fields showed sluggishly motile organisms, while very few colonies were obtained in the Endo plates. In a concentration of 1:125,000 the cells were very sluggish, but an abundance of growth was obtained on the Endo agar. In 1:100,000 concentration "only Brownian movement" was seen in the few organisms observed, but isolated colonies of the typhoid bacillus were easily obtained on the Endo plates, though in small numbers.

No explanation is offered here regarding the immediate cause of loss of motility in the higher concentrations of brilliant green, though subculture tests have shown that the organisms which become nonmotile are not necessarily killed or permanently injured.

The loss of motility may have a direct bearing on the selective action of the medium. In practice, loop transfers are made from the fluid far above the sediment which contains the bulk of colon bacilli and other nonmotile or sluggishly motile organisms present, along with the solid particles of feces, etc. When the proper proportion of dye is used to materially inhibit *Bact. coli* and at the same time to allow the typhoid organism to grow and retain its motility, transfers made from the supernatant fluid should contain this organism in relatively large numbers and practically pure, and result in good isolations on the Endo plate. Motility, therefore, may be a distinct aid in the isolation of *Bact. typhosum*.

ROUTINE ISOLATION OF THE TYPHOID ORGANISM BY THE USE
OF THE IMPROVED (BUFFERED) BRILLIANT GREEN ENRICH-
MENT MEDIUM AND THE MODIFIED ENDO MEDIUM

The problem of isolating these organisms from the feces of typhoid patients and of carriers is of paramount importance. This problem has been particularly acute insofar as carrier identification is concerned, because of the small numbers of these organisms which as a rule occur in the feces. Most laboratories do not depend on any one medium. The usual procedure is to employ two or three of the more widely used mediums in the hope that at least one will yield the desired results.

The enrichment method used in this investigation is simple and has proven in our hands to be surprisingly efficient, as compared with others. It has been applied by us in the examination of a large number of artificially infected stools as well as feces from known clinical cases.

The four mediums which appear to be most commonly employed by the various laboratories for the isolation of *Bact. typhosus* are Endo agar, brilliant green agar, eosin methylene blue agar and brilliant green bile. In order to gain more definite information on the choice of mediums a questionnaire was sent to fifty of the leading hospitals and state laboratories in the country. Replies received from 31 laboratories show that 31 made examinations of feces and urine; 30 used Endo medium, either alone (21) or combined with the use of some other medium; 4 used brilliant green agar, but also Endo medium as a check; 11 used eosin methylene blue, but 7 of them also used Endo medium for plating; 3 employed brilliant green bile, and 1 used brilliant green broth as an enrichment fluid, and Endo medium for plating.

The widespread use of Endo agar further confirmed our belief in the value of this medium for typhoid and paratyphoid isolation. The Robinson and Rettger⁴ modification was chosen here because it gives such a sharp contrast between lactose fermenters and nonfermenters, as Krumwiede, Kahn and Kuthner,¹⁷ and Gilbert and Coleman¹⁸ have shown, and because it is definitely standardized and remains quite stable for reasonably long periods in storage. Endo agar prepared by this method has been kept in the laboratory refrigerator for a period of five weeks without marked deterioration.

Brief Details of the Method.—A peptone solution, 2%, is prepared (20 gm. peptone, 5 gm. NaCl in 1000 cc. water). The reaction is so adjusted that the

¹⁷ J. Infect. Dis., 1918, 23, p. 275.

¹⁸ Abstr. Bact., 1922, 6, p. 35.

medium has a H-ion concentration of P_H 6.5 after sterilization. In our experience of pre-sterilization reaction of P_H 6.1 was found to furnish the desired final point. The peptone water is distributed in 6 x $\frac{3}{4}$ inch tubes, 5 cc. per tube, just before autoclaving at 115 C. for 15 minutes.

The buffer mixture used here is phosphate solution 2 previously described. It consists of 7.5 grams K_2HPO_4 and 7.5 grams KH_2PO_4 dissolved in 155 cc. water. This solution is sterilized at 115 C. for 15 minutes, and constitutes the stock buffer solution, with a H-ion concentration of P_H 6.5. Of this stock solution 0.4 cc. (1%) is added aseptically to each of three tubes of the peptone water. The stock solution of brilliant green (Grübler's or some other certified brand) consists of 1% dye in distilled water. Immediately before use a 1:10,000 dilution is prepared (0.1 cc. of the stock solution plus 9.9. cc. distilled water). The following amounts of this dilution are then added respectively to three tubes of the peptone water: 0.3 cc.; 0.2 cc.; and 0.1 cc. This yields final dye dilutions of approximately 1:165,000, 1:250,000 and 1:500,000 strengths.

A 4 mm. platinum loop is used for inoculating the enrichment medium with the feces, and later for transfer to the Endo plate. For the streaking of the Endo agar a platinum needle bent at an angle of 120 C. and with a flat "streaking tip" about $\frac{3}{4}$ inch long was employed. The loopful of brilliant green broth culture is placed on the Endo plate and streaked gently over the entire agar surface with the wire. A bent glass rod may be used in place of the wire.

Experiments with Artificially Infected Feces.—This series of fecal examinations covered a period of over two years, and involved the use of 204 specimens. Grübler's brilliant green (cryst.) was employed in every experiment. The samples of feces were inoculated as follows:

One-half gram of the fresh sample was rubbed up in 5 cc. distilled water; this material in the form of a thick emulsion or mash was then inoculated with very small amounts of an 18 hour peptone water culture of *Bact. typhosum*. The entire material was thoroughly mixed and allowed to stand for about five minutes, to allow the coarse particles to settle out.

A 4 mm. loopful of this artificially infected fecal material was inoculated into each of the three tubes of brilliant green buffered peptone water. For control, a plain peptone tube was inoculated with the same amount of material, and Endo plates were streaked directly. The tubes were incubated for 18 to 24 hours at 37 C, and the control plates for 18 hours at 34 C. A 4 mm. loopful from each of the four incubated tubes was streaked over the surface of Endo medium. These plates were incubated for 18 hours at 34 C. and examined for the presence of *Bact. typhosum*.

In all of these 204 individual experiments with artificially infected feces the typhoid bacillus was never observed on the control (directly

inoculated) plates. In this manner the superiority of the modified enrichment medium over direct plating was clearly demonstrated.

In order to make certain that the specimens contained *Bact. coli*, more than half of the 204 specimens were inoculated also with an 18 hour culture of this organism.

All of the strains of *Bact. typhosum* used here were recently isolated ones, except Rawlins, Hopkins, and strains 3, 19 and 30. The results are shown in table 4.

These results are of prime significance, especially when the fact is taken into consideration that in not one instance was *Bact. typhosum* recovered from the Endo plates which were streaked directly with the

TABLE 4
RESULTS OBTAINED WITH ARTIFICIALLY INOCULATED FECES

Strain	Number of Specimens Examined	Number Positive with Brilliant Green Enrichment*	Percentage Positive Isolations
<i>Bact. Typhosum</i>			
Hopkins.....	24	22	92
Rawlins.....	14	14	100
3.....	19	17	90
19.....	11	11	100
30.....	15	15	100
C.....	15	12	80
S.....	9	9	100
R.....	10	7	70
N.....	7	7	100
L. H.	24	21	87
<i>Bact. paratyphosum</i> A.....	30	29	97
B.....	26	26	100
Total.....	204	190	93

* Controls—plain peptone water inoculated and subcultured directly on Endo plates—were all negative for typhoid bacteria.

fecal suspensions. These control plates were covered with *Bact. coli* colonies to such an extent as to make isolation of the typhoid bacillus impossible, even if it was present. On the other hand, there were many instances where plates streaked with material from the brilliant green tubes presented the picture of pure or almost pure *Bact. typhosum* plates.

As a rule, none of the three brilliant green dilutions used gave much better results than the other two. All three often yielded abundant typhoid colonies. The more typical plates contained some 12 to 15 large, red coli colonies interspersed among a few hundred characteristic, small colonies, and as a rule, well isolated colonies of the typhoid bacillus.

In the transfer of material from the brilliant green enrichment tubes to Endo agar, a 4 mm. loopful was always taken from the upper half of

the fluid of those tubes which showed a faint cloudiness, without previous shaking. In instances where the tubes remained clear but possessed a slight sediment, the tubes were shaken lightly and allowed to stand for a minute or two before the transfer was made.

In quite a number of occasions the dye was thrown out of solution in the 1:500,000 dilution. This was probably due to absorption of the dye by particles of suspended matter, and not alkalization of the medium. In such instances, however, satisfactory isolations were made from one or both of the other enrichment tubes.

Examination of Normal and Artificially Inoculated Fecal Specimens. Bacterial Content Unknown to the Operator.—In this phase of the present investigation a comparative study was made of the efficacy of the combined buffered brilliant green enrichment medium and Endo agar, and the eosin methylene blue agar of Holt-Harris and Teague.¹⁹

The so-called "unknown" specimens were those of normal feces, some of which were artificially inoculated with minute amounts of known typhoid culture, while others were left untreated. These test samples were prepared as follows, by a person apart from the isolation technician. Each day one or more of four fresh fecal suspensions supplied for the purpose were inoculated with minute portions of an 18 hour broth culture of *Bact. typhosum*. When three or four specimens were inoculated a very small loopful of the typhoid culture was introduced into the first tube. The same loop was then withdrawn and applied to the other tubes in series, without the use of any additional fecal suspension. In some instances the tips of platinum wires were employed in place of the loop.

Both Grüber and the "standard" C & B samples of brilliant green were used, three dilutions of the dye being used for each specimen, as described previously in this paper. The eosin methylene blue agar used was Difco dehydrated agar.

A series of six tubes were inoculated from each "unknown" specimen, three containing dilutions of the accepted C & B, and three of the Grüber samples of dye, and the entire process carried out as in the preceding series of tests on the 204 artificially infected fecal specimens. In addition, two eosin methylene blue and one Endo plate were streaked directly from each of the "unknowns." All of the colonies "fished" as

¹⁹ J. Infect. Dis., 1916, 18, p. 596-600.

typhoid were further identified by inoculation of Russell's medium,²⁰ and glucose and lactose broth. The results of the examinations were as follows:

Number of unknowns examined.....	56
Number of unknowns actually inoculated.....	39

Medium	Isolation of Typhoid Organisms	
	Number	%
Endo	12	28
Eosin methylene blue.....	7	17.5
Brilliant green and Endo.....	37	92.5

The advantages of the use of the enrichment medium are clearly shown here, this method giving positive findings, in 92.5 of the inoculated specimens, against 28% for the direct Endo plating and 17.5% for the eosin methylene blue method. Several typhoid-like colonies fished from the eosin methylene blue plates gave confirmatory reactions for *Proteus vulgaris*. Furthermore, the contrast between the lactose fermenters and nonfermenters was much sharper on the Endo than on the eosin methylene blue agar.

Examination of Feces from Typhoid Patients (Clinical Diagnosis) and Suspects.—During the epidemic of typhoid fever in New York City in the winter of 1925-26 an opportunity was afforded to apply the present method of isolation in clinical cases.

With one or two exceptions, all of the fecal specimens examined were from patients who were not ward cases, but were under the care of their own physicians and nurses. For this reason the number of stools received was limited. All specimens were examined within four hours after their receipt. In the case of formed stools, one-half gram of feces (mass of about the size of a large pea) was emulsified in 5 cc. sterile water and the suspensions (4mm. biconvex loop) introduced into the enrichment medium. When the feces were of a diarrheal character a 4 mm. loopful was transferred directly to the brilliant green peptone water tubes.

The mediums and method employed here were the same as previously described. Eosin methylene blue and Krumwiede's brilliant green agar plates were streaked directly in several instances, while Endo agar was streaked in all cases, for purposes of comparison. Shortage of Petri dishes prevented the use of all three plated mediums throughout

²⁰ J. M. Res., 1912, 25, p. 217.

the experiments. Typhoid-like colonies were inoculated into Russell's double sugar medium and into glucose, lactose and xylose broth, and the typical isolated organisms subjected to the macroscopic and microscopic agglutination tests. A general summary of the results is given in the following tabulations.

POSITIVE ISOLATIONS OBTAINED. COMPARISON OF BUFFERED BRILLIANT GREEN ENRICHMENT WITH DIRECT PLATING METHODS

Number of cases.....	13
Isolations: by direct plating, Endo.....	5 (38.5%)
by brilliant green enrichment and Endo	11 (84.6%)

NUMBER OF POSITIVE ISOLATIONS OBTAINED WITH VARIOUS MEDIUMS

Number of specimens examined.....	50 *
Isolations: by Endo, direct.....	5
by Eosin methylene blue.....	0
by brilliant green agar.....	0
by brilliant green enrichment.....	22

*NUMBER OF SPECIMENS OBTAINED IN EACH CASE AND RESULTS OF EXAMINATION

Case	Number of Specimens Examined	Isolations	
		By Endo Alone	By Enrichment Medium and Endo
1.....	6	0	0
2.....	7	1	2
3.....	8	1	5
4.....	6	0	2
5.....	3	0	2
6.....	1	0	1
7.....	1	0	1
8.....	1	0	0
9.....	1	0	1
10.....	6	0	3
11.....	2	0	1
12.....	2	0	1
13.....	3	3	3
Total.....	50	5	22

NUMBER OF TIMES VARIOUS MEDIUMS WERE USED AND ISOLATIONS OBTAINED WITH EACH

Endo, direct.....	50	5 (10%)
Eosin methylene blue.....	15	0
Brilliant green agar, direct.....	14	0
Brilliant green enrichment and Endo....	50	22 (44%)

The advantages of the buffered brilliant green enrichment and Endo plating method, at least in the writers' hands, over the other mediums employed here are clearly shown in these summaries.

Of the 50 fecal specimens from clinical cases, 22 or 44%, were found to be positive by this combination method, while by direct plating on Endo medium only 5, or 10%, yielded positive findings, and the limited numbers of attempts to isolate on the eosin methylene blue and brilliant green agar plates, none were successful.

Positive isolations were made from the feces of eleven out of 13 clinical typhoid cases. One of the two patients which gave negative results, had experienced a return to normal temperature from ten to twelve days previous to the test. In the case of the other negative patient, only one specimen was examined, and this under adverse conditions, that is after a considerable period after voiding.

SUMMARY

The buffered brilliant green enrichment medium and modified Endo agar, as combined and employed in the present investigation, have given results far more satisfactory than direct plating on Endo agar, on eosin methylene blue, or on brilliant green agar. The examination of 204 artificially infected specimens over a long period of time, and of over 50 "unknowns" and 50 samples from clinical cases has demonstrated the value and the possibilities of the combined methods (buffered brilliant green broth and Endo medium) in the isolation of *Bact. typhosum* from feces and in the identification of typhoid cases and carriers.

The most favorable dilutions of the brilliant green range from 1:165,000 to 1:500,000. The three dilutions employed here, 1:165,000, 1:250,000 and 1:500,000, appeared to satisfy the conditions of the test.

These dilutions are unfavorable for *Bact. coli* growth, and allow the typhoid bacillus, when present, to develop in large numbers, in the presence of sufficient phosphate buffer to keep the H-ion concentration within P_H 7.0. However, on the Endo and eosin methylene blue plates streaked directly the growth was chiefly that of *Bact. coli*, and the typhoid bacillus was rarely observed.

The success of the present combination of methods must be attributed in a large measure to the strong buffering agent—mixed phosphate solution—which prevents the peptone water from becoming alkaline and throwing the brilliant green out of solution.

In practically all of the successful tests Grüber brilliant green was employed. Only one of the six American brands used gave results at all comparable to those obtained with the Grüber brand.

Endo medium serves as a very efficient plating and isolation medium, particularly the modified medium of Robinson and Rettger. The excellent contrast observed between the colonies of lactose fermenting and nonfermenting organisms furnishes an easy means of isolation of *Bact. typhosum* (or of *Bact. coli*, as in the sanitary examination of water, and the writers believe it offers advantages superior to those of eosin methylene blue and brilliant green agar.

Bact. alkaligenes and some strains of *Bact. aerogenes*, when present, may cause considerable annoyance, and perhaps failure, but fortunately these two organisms are found only very occasionally in feces.

The writers would strongly urge that persons contemplating the use of the modified brilliant green Endo method in routine examination familiarize themselves first with the technic by carrying out a number of experiments on samples of feces which are known to contain typhoid or paratyphoid bacilli.

Particular attention must be given to the amount of feces introduced into the tubes of brilliant green broth; also to the streaking of Endo plates from the incubated enrichment tubes. As has been stated elsewhere in this paper, transfers from brilliant green peptone water which possess some cloudiness should be made, without shaking, from the upper half of the medium. Tubes which are clear, except for a precipitate, should be shaken, and allowed to settle for one or two minutes before similar transfers to the Endo medium are made.

STUDIES ON ANTIGEN FOR THE KAHN TEST

UNIFORMITY IN SENSITIVENESS OF STANDARD ANTIGEN

R. L. KAHN, NATHAN NAGLE AND PEARL L. KENDRICK

From the Bureau of Laboratories, Michigan Department of Health, Lansing

A more thorough understanding of the phenomenon of precipitation with syphilitic serum will come when the chemical nature of the antigen is better understood. This, indeed, is one of the ultimate objectives of this series of studies—to identify the substance or substances in the antigen causing precipitation. Meanwhile, however, it seemed necessary to extend our knowledge of the general properties of antigen and thereby gain greater control of the precipitation test, both as a research and as a diagnostic agent.

During the past few years, data have been collected in this laboratory on several phases relating to standard antigen employed in the Kahn test. These deal with changes in beef heart and their effect on the sensitiveness of the final antigen product, also with factors affecting the standardization of the antigen. This article will consider some of these phases and especially the problem of uniformity of antigen sensitiveness in the Kahn test. Uniformity in sensitiveness of antigen is obviously of major importance in any immunologic or biologic method. In serologic tests for syphilis many difficulties have stood in the way of producing uniform antigens. Heart tissue, which is the base of such antigens, appears to vary in the composition of specific lipoids. It is common knowledge that two antigens prepared from different beef hearts by the same method, may vary in sensitiveness. The diet of the beef and the extent of autodigestion of the heart muscle may play important rôles in determining the sensitiveness of an antigen. In order to overcome differences in sensitiveness, various methods of antigen titration have been devised. These depend on the dilution of the antigen with greater or lesser amounts of physiologic salt solution. But these titration methods do not always bring an antigen to the required sensitiveness. It is not unusual among serologists to find it necessary to discard an antigen due to its lack of sensitiveness, although prepared according to standard requirements.

Received for publication, April 18, 1927.

The method for preparing and titrating antigen for the Kahn test was standardized early in 1924.¹ Since that time, the test has been favorably reported upon by a host of workers, corroborating our findings with regard to the sensitivity of the antigen. It has been recognized however, that certain lots of beef heart might vary to such an extent that the simple titration method based on dilution with salt solution would not be adequate to insure uniform sensitiveness of the antigen. In order to study this problem we experimentally produced changes in beef heart, made standard antigen from the varying lots and attempted to determine whether a method could be devised for bringing all these varying antigens to a uniform degree of sensitiveness. Our findings indicate that if Kahn antigen is made according to specifications it is possible in practically every case to correct it to a degree of sensitiveness uniform with a standard, as will be shown by experiments presented in this article.

In order to simulate conditions that might exist in the preparation of different antigens, we modified beef heart by incubating it for varying periods, thus allowing varying degrees of autodigestion to take place.

Sensitiveness of Antigen. Effect of Incubating Wet Heart Muscle at 37 C.—A uniform mixture of wet ground beef heart was divided into four parts. Part 1 was dried in a vacuum drying oven immediately after grinding, while parts 2, 3 and 4 were incubated at 37 C. for 5, 10 and 15 hours, respectively, before drying. Each fraction of the dried beef heart was ground into powder form and used in the preparation of standard Kahn antigen. Antigens prepared from beef heart lots 1 to 4 were tested for sensitiveness employing weakly positive syphilitic serums. It was observed that comparatively short periods of autodigestion of beef heart have little effect on antigen sensitiveness. When the autodigestion was extended to 15 hours, however, the beef heart produced antigen of somewhat greater sensitiveness. The reason for this is not clear to us. It is possible that autodigestion, by disrupting the protein molecules, permits greater extraction by alcohol. Based on this possibility, an attempt was made to prepare antigen from proteose and peptone obtained on the market. It was found, however, that both of these ingredients underwent semicoagulation in the alcohol, and the final extract was practically free from antigenic substance.

Sensitiveness of Antigen. Effect of Incubating Dry Powdered Heart Muscle at 37 C.—A portion of a lot of powdered beef heart was placed in the incubator at 37 C. for one month. Antigen was prepared from it and from the same heart muscle which was not incubated, and both tested with weakly positive syphilitic serums, employing standard antigen as a control.

The results recorded in table 1 indicate that incubating powdered beef heart for a month, hastening presumably autodigestion of the heart muscle, tends to increase antigen sensitiveness. On extending the incubation period an additional month, slightly greater antigen sensitiveness

¹ Serum Diagnosis of Syphilis by Precipitation, 1925.

was observed. When incubation was prolonged to six months or a year, the beef heart in some cases was found to be unfit for the preparation of antigen. An antigen made from such beef heart showed a tendency to produce an insoluble precipitate in mixtures of the antigen and salt solution.

Necessity for Testing the Sensitiveness of a New Antigen with Serums.—Recognizing that autodigestion and possibly other changes in beef heart are likely to affect the sensitiveness of antigen, it was believed that the standard titration method might not always bring antigens to a uniform degree of sensitiveness. Two antigens, for example, might each have a titer of 1 cc. antigen + 1 cc. normal salt solution and still

TABLE 1

EFFECT OF INCUBATING HEART MUSCLE FOR ONE MONTH AT 37 C. ON ANTIGEN SENSITIVENESS

Serums	Standard Antigen Titer 1 + 1.1			Antigen Prepared From Dried Beef Heart Not Incubated Titer 1 + 1			Antigen Prepared From Dried Beef Heart Incubated One Month at 37 C. Titer 1 + 1		
	1	2	3*	1	2	3	1	2	3
1-3	++++	++++	++++†	++++	++++	++++	++++	++++	++++
4	+	++++	++++	0	+++	++++	+	++++	++++
5	0	+++	++++	0	±	++	0	+++	++++
6	0	±	+++	0	±	++	0	+	++++
7	0	++	+++	0	±	++	0	+	+++
8	0	±	++	0	0	0	0	±	+
9	0	+	++	0	±	+	0	+	++
10	0	+	++	0	+	++	0	+	++
11	0	0	+	0	0	0	0	±	+
12-20	0	0	0	0	0	0	0	0	0

* In this and subsequent tables results are presented as obtained with the regular three-tube Kahn test.

† +++++ = strong precipitation; +++, ++, +, ± = progressively weaker precipitation, and 0 = no precipitation.

show some variation in sensitiveness. Antigen standardization, therefore, was divided into two steps: first, the determination of the titer with normal salt solution; second, the determination of the degree of sensitiveness with syphilitic serum employing standard antigen as a control.

It is emphasized that variation in sensitiveness between different antigens must be determined by employing weakly positive syphilitic serums. Strongly positive (++++) serums would show, in most cases, no variations. It is suggested that the weakly reacting serums be prepared by diluting strongly positive serums with negative serum to the point where they give a reaction with standard antigen of approximately negative, two and four plus, respectively, in the three tubes of the Kahn test. Preliminary tests should be made with standard antigen to determine that the reactions are as desired. Six serums with such

reactions, two four plus and two negative serums make a convenient number for the first comparative tests of the new antigen with the standard.

Varying Titers of Kahn Antigen.—The method of titration of antigen which determines the amount of physiologic salt solution to add to 1 cc. antigen, tends to equalize antigen sensitiveness. As was shown elsewhere, increasing the amount of salt solution for antigen dilution tends to decrease sensitiveness while decreasing salt solution tends to increase sensitiveness. The titration is so adjusted that an antigen rich in specific lipoids requires for its dilution a larger amount of salt solution than an antigen poor in lipoids. Thus, the titer of an antigen rich in lipoids might be 1 cc. antigen + 1.5 cc. salt solution, while the titer of an antigen poor in lipoids might be 1 cc. + 0.8 cc. saline. This assures relative uniformity of lipoid content in the final antigen-salt solution mixture which is employed with serum.

During the past two years we have had antigens which varied in their titer from 1 cc. antigen + 0.8 cc. salt solution, to 1 cc. antigen + 1.8 cc. salt solution. These antigens were sent to us from different biological, public health and hospital laboratories with a view of assisting in their standardization. Of 60 antigens thus submitted from July, 1925, to January, 1927, only two were found to be impossible to correct and therefore unfit for use. These two antigens gave insoluble precipitates after the addition of salt solution within a wide range, and it was believed that they were not prepared according to standard requirements. Of the remaining antigens, 56 showed titers ranging from 1 + 1 to 1 + 1.8, while the remaining two antigens showed titers of 1 + 0.8. In most cases, these antigens gave results with syphilitic serums similar to the standard antigen prepared in this laboratory. Some antigens showed differences in sensitiveness and thus required correction.

Reduction of Amount of Salt Solution in Titer of Antigen.—In attempting to bring antigens having varying titers to the required sensitiveness, it seemed advantageous first to reduce them to approximately the same titer of 1 cc. antigen plus 1.1 cc. physiologic salt solution. As already indicated, the reason certain antigens required higher titers than 1 + 1.1 is undoubtedly because such antigens contain an excessive amount of beef heart lipoids. It was believed that the addition of alcohol in proper amounts to such antigens would sufficiently reduce the lipoid concentration to bring their titer to 1 + 1.1. In diluting cholesterolized antigen, cholesterolized alcohol containing 0.6% cholesterol would be employed so as not to change the % of cholesterol con-

tent in the final antigen. Preliminary experiments soon indicated that by diluting an antigen with cholesterolized alcohol, titers as high as $1 + 1.8$ could readily be reduced to $1 + 1.1$. Of greater importance than the mere reducing of antigen titer to $1 + 1.1$, however, was the determination as to what effect this reduction had on antigen sensitiveness. This could be readily gauged by testing the sensitiveness of the diluted antigen in comparison with the standard antigen. By employing the diluted antigens in a $1 + 1.1$ titer in these comparative tests, such antigens would thus be standardized at this titer.

It should be emphasized that in the standardization of an antigen, the final criterion is the degree of sensitiveness with syphilitic serum and not the amount of normal salt solution specified in the titer. Thus, an antigen having a titer of $1 + 1.2$ or $1 + 1.3$ giving results with serum comparable to the standard does not need dilution with alcohol in order to reduce the titer to $1 + 1.1$. Even such antigens which require excessive amounts of salt solution for antigen dilution such as $1 + 1.8$ may safely be used without dilution with alcohol, provided the results with serum are the same as with standard antigen. The utilization of alcohol dilution is especially valuable in the case of antigens giving results at variance in sensitiveness with standard antigen. Thus, an antigen may have a titer of $1 + 1.5$ and at this titer give results with serum different from standard. In diluting such an antigen with alcohol our aim was to employ, purely for reasons of uniformity, a $1 + 1.1$ titer.

The Standardization of Antigens Having Titers of $1 + 1.1$ to $1 + 1.8$.—The following outline presents the scheme employed in these laboratories in standardizing antigens having a $1 + 1.1$ or higher titer to uniform sensitiveness.

Antigens Titrating $1 + 1.1$.—If a newly prepared antigen gives a titer of $1 + 1.1$, its sensitiveness is checked against that of standard antigen in use, employing ten serums most of which are weakly positive. The latter can be prepared artificially, as already indicated, by diluting strongly positive serums with negative serum. An illustration of this method of checking antigen sensitiveness is presented in table 2, in which antigen lot 5, newly prepared, was checked against lot 4, the standard antigen in use. Small variations between the readings of individual tubes are to be expected. These variations become negligible when the final result which is the average of the findings of the three tubes is considered.

If the new antigen proves to be more sensitive than the standard, the principle of reducing the sensitiveness by increasing the amount of physiologic salt solution in the antigen dilution is utilized. Thus, instead of employing a $1 + 1.1$ titer, a $1 + 1.2$ or $1 + 1.3$ titer is used. The antigen with either or both of these titers is checked against standard antigen employing the usual number of weakly positive syphilitic serums. The titer at which the sensitiveness of the new antigen is practically identical with the sensitiveness of the standardized antigen, is accepted

as the new standard. In our experience we have never had occasion to change a 1 + 1.1 titer to as high as 1 + 1.3. But we did find it necessary to change a 1 + 1.1 to 1 + 1.2 titer to bring it to identical sensitiveness with the standard. Then again there is the possibility that an antigen giving a 1 + 1.1 titer is somewhat less sensitive than the standard. In the one case in which we observed this to be true, we found that adding ten parts of cholesterolized alcohol (0.6%) to 100 parts of the cholesterolized antigen and using the diluted product in a 1 + 1.1 titer, increased the sensitiveness of the antigen to standard requirements.

It should be pointed out in this connection that the dilution of antigen with alcohol may in one case increase and in another decrease the sensitiveness of an antigen. Experimental data throwing light on the probable reason for this will be presented elsewhere. Our purpose here is merely to present the findings of our attempt to maintain a uniform scale of sensitiveness of standard Kahn antigen.

Antigens Titrating 1 + 1.2.—A newly prepared antigen giving a titer of 1 + 1.2 may check in sensitiveness with the standard antigen, which renders it ready for use. The new antigen may be somewhat more sensitive than the standard antigen.

TABLE 2
SENSITIVENESS OF FRESHLY PREPARED ANTIGEN LOT 5 COMPARED WITH STANDARD ANTIGEN LOT 4

Serums	Antigen 4 Titer 1 + 1.1				Antigen 5 Titer 1 + 1.1			
	1	2	3	Final Results	1	2	3	Final Results
1	++++	++++	++++	++++	++++	++++	++++	++++
2	+++	++++	++++	++++	++	++++	++++	+++
3	+	++++	++++	+++	++	++++	++++	+++
4	±	+++	++++	++	0	++	++++	++
5	±	+++	+++	++	0	+++	++++	++
6	±	+	++++	++	0	++	+++	++
7	0	±	+++	+	0	+	++	+
8	0	±	++	±	0	0	++	±
9	0	±	+	0	0	±	±	0
10	0	0	0	0	0	0	0	0

In that case, increasing the titer to 1 + 1.3 or 1 + 1.4 may bring the antigen to standard sensitiveness. Then again the new antigen may be somewhat less sensitive than the standard, in which case dilution with cholesterolized alcohol is resorted to. As in the case of the antigen giving a titer of 1 + 1.1 discussed above, 10% dilution with cholesterolized alcohol may bring the antigen to proper sensitiveness. If still less sensitive, 20% dilution undoubtedly will be ample.

Antigens Titrating 1 + 1.3 to 1 + 1.8.—We have not seen an antigen requiring a titer higher than 1 + 1.8, but there is no reason why such an antigen might not be of standard sensitiveness or be corrected to such a sensitiveness. Antigens giving titers of 1 + 1.3 to 1 + 1.8 have been corrected in this laboratory by dilution with cholesterolized alcohol. By this means the antigens have been reduced to a titer of 1 + 1.1 and to a degree of sensitiveness equal to that of standard antigen. As indicated, dilution with cholesterolized alcohol will bring antigens which are either less sensitive or more sensitive than the standard to the required sensitiveness. This is illustrated, experimentally, in tables 3 and 4.

Table 4 gives the results of antigen 8 compared with control antigen 7 employing ten serums. The titer of antigen 8 was 1 + 1.3. At this titer the antigen was

TABLE 3

ANTIGEN HAVING TITER 1 + 1.3 AND LESS SENSITIVE THAN STANDARD ANTIGEN, CORRECTED BY 15%
DILUTION WITH CHOLESTEROLIZED ALCOHOL

Serums	Antigen 8								
	Antigen 7 Standard Antigen Titer 1 + 1.1			Undiluted Titer 1 + 1.3			15% Dilution Titer 1 + 1.1		
	1	2	3	1	2	3	1	2	30% Dilution Titer 1 + 1.1
1	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+
3	0	+	+	+	+	+	0	+	+
4	0	+	+	+	+	+	0	+	+
5	0	+	+	+	+	+	0	+	+
6	0	+	+	+	+	+	0	+	+
7	0	+	+	+	+	+	0	+	+
8	0	+	+	+	+	+	0	+	+
9	0	+	+	+	+	+	0	+	+
10	0	+	+	+	+	+	0	+	0

TABLE 4

ANTIGEN HAVING TITER OF 1 + 1.7 AND MORE SENSITIVE THAN STANDARD ANTIGEN
CORRECTED BY 30% DILUTION WITH CHOLESTEROLIZED ALCOHOL

Serums	Antigen B								
	Antigen 9 Standard Antigen Titer 1 + 1.1			Undiluted Titer 1 + 1.7			15% Dilution Titer 1 + 1.1		
	1	2	3	1	2	3	1	2	30% Dilution Titer 1 + 1.1
1	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+
6	0	+	+	+	+	+	+	+	+
7	0	+	+	+	+	+	+	+	+
8	0	+	+	+	+	+	+	+	+
9	0	+	+	+	+	+	+	+	+
10	0	+	+	+	+	+	+	+	+

somewhat less sensitive with syphilitic serum than the standard. On diluting this antigen with 15 and 30%, respectively, cholesterolized alcohol, it was found that with the former dilution, the antigen gave results similar to the standard, while with the latter dilution it gave results weaker than the standard. It is clear from this table that the addition of 15 parts of cholesterolized alcohol to 100 parts of antigen 8 increased its sensitiveness to a degree to class it as standard.

Table 4 gives the comparative results between standard antigen and one giving a titer of $1 + 1.7$ (antigen B). As is evident from the table, this antigen in a titer of $1 + 1.7$ gave results which were somewhat more sensitive than the standard. On diluting this antigen with 15, 30 and 45% cholesterolized alcohol, respectively, the 30% dilution was found to give results comparable with the standard.

Technical Considerations.—For standardizing antigens having titers of $1 + 1.1$ or higher, a stock solution of cholesterolized alcohol is repared by dissolving 0.6% cholesterol in 95% alcohol and filtering. When preparing test dilutions of antigen with cholesterolized alcohol for check purposes, 5 cc. amounts of antigen are employed. Thus, 0.75 cc. and 1.5 cc. amounts of cholesterolized alcohol are added to 5 cc. antigen amounts, respectively, resulting in 15 and 30% dilutions which can then be used with a titer of $1 + 1.1$ in comparison with standard antigen employing weakly positive syphilitic serums. For example, should a 15% dilution check with standard, then the entire amount of available antigens diluted with 15% cholesterolized alcohol. More specifically, to 100 parts cholesterolized antigen are added 15 parts cholesterolized alcohol. To make sure that no error entered in making the dilution, it is well to check again the diluted product against standard antigen with weakly positive serums.

The Standardization of Antigens Having Titers Less Than $1 + 1.1$.—In our experience this group of antigens contains but two classes: those having titers of $1 + 1$ and $1 + 0.8$. The reason for these "low" titers is believed to be the meager lipid content of the antigen. Should antigens having either of these titers give results with serums comparable to standard antigen, then no further standardization is necessary. Should such antigens give results more sensitive than the standard, their sensitiveness could be readily reduced to the required degree by increasing the amount of salt solution for antigen dilution. Thus, an antigen giving a titer of $1 + 0.8$ or $1 + 0.9$ might be employed in a titer of $1 + 1$ or $1 + 1.1$. Comparative tests with standard antigen using weakly positive serum will indicate the titer which should be considered standard for a given antigen.

There is still a third possibility, namely, that antigens giving low titers may prove less sensitive than the standard. Since this is undoubtedly due to the insufficient concentration of antigenic lipoids, the basis for correcting such antigens is to increase the concentration of these lipoids. Our experience indicates that increasing the lipid concentration with either alcohol or ether extractives of beef heart will give equally good results. The method of lipid concentration by adding ether

extractives to antigen has already been discussed elsewhere.¹ Briefly, the steps involved are as follows:

The ether extracts obtained from the preparation of Kahn antigen from 25 grams of beef heart are collected and evaporated to about 50 cc. This concentrated ether extract is cleared by filtration and kept as stock solution. To two 5 cc. amounts of the weak antigen are added, respectively, 0.025 and 0.05 cc. amounts of the ether extract, producing 0.5 and 1% concentrations. These modified antigens in titers of 1 + 1.1, are tested with weakly positive syphilitic serums, using standard antigen as a control. The antigen which gives results closely comparable to the standard is established as the new standard and the entire amount of antigen is accordingly concentrated by adding the indicated amount of the ether extract. If neither 0.5 nor 1% concentration gives desired results, then 0.75% concentration might produce an antigen comparable to the standard. We have not had occasion to use more than 1% concentration with the ether extract.

The only time we were called upon to correct antigen by this method, was in July, 1925. The titer of the antigen was 1 + 0.8; with negative serum it gave reactions which were extraordinarily clear—almost like water, while with syphilitic serum it gave reactions considerably weaker than the standard antigen control. By preparing a concentrated ether extract, as described above, and adding 0.025 and 0.05 cc. amounts respectively, to two 5 cc. quantities of the antigen, it was found by testing the final products with serum that 0.05 cc. (1%) was too large an amount to add to the antigen, the negative serums appearing cloudy instead of opalescent and clear while 0.025 cc. of the ether extract was insufficient an amount, as shown by the fact that the final antigen was not quite so sensitive as the standard. To an additional 5 cc. amount of unconcentrated antigen was then added 0.0375 cc. of ether extract (0.75%). The final antigen in this case was found to give results similar to standard antigen. To two liters of antigen were accordingly added 15 cc. of the ether extract. The final product was thoroughly mixed and filtered after standing 24 hours at room temperature to remove the trace of insoluble material. On rechecking the sensitiveness of this antigen with serums against standard antigen, the results showed close parallelism and the product was thus approved as standard antigen.

Antigens corrected by concentration with ether extractives show a tendency to throw down a slight precipitate on prolonged standing. This precipitate consists of lipoids which are apparently highly soluble in ether and poorly soluble in alcohol. On placing the antigen flask in warm water, this precipitate will almost entirely redissolve. If the trace of insoluble material is removed by filtration, it is often found that no further precipitate is formed. Should it form, however, rewarming and refiltering will often clear it permanently.

As was stated, antigens containing insufficient lipoids as indicated by low titer and insufficient sensitiveness may also be corrected by increasing the concentration of the alcohol extractives. It was observed that a noncholesterolized antigen may be diluted with a given amount of alcohol and reconcentrated by removing this alcohol by evaporation without affecting the titer or sensitiveness of the antigen after cholesterolization.

This observation led us to utilize this concentration method in correcting an antigen of a $1 + 0.8$ titer (antigen L) and giving unusually weak reactions with syphilitic serums. The method of concentration was as follows:

Into three small evaporating dishes were added 2.5, 5 and 7.5 cc. amounts, respectively, noncholesterolized Kahn antigen and evaporated almost to dryness. Each of these antigen residues were dissolved in 10 cc. amounts of cholesterolized antigen L. There were thus available, aside from the original unconcentrated antigen L, three antigens, one having additional concentration of 25%, another 50%, and the third 75% of alcoholic extract lipoids. These four antigens using a $1 + 1.1$ titer were then tested with weakly positive syphilitic serum employing standard antigen as a control. The results of the comparative study are presented in table 5.

It is clear from this table that 50% concentration of antigen L by means of alcoholic extractives gave results practically identical with the standard; that 25% was insufficient and 75% was excessive concentration. On repeating the 50% concentration experiment the results were again practically like the standard control.

By the methods described in this paper, antigen may be brought to a definite degree of sensitiveness with syphilitic serums, using a known standard antigen as the basis of comparison. In order, the steps of the procedure are: preparation of antigen according to specifications,¹ determination of titer,¹ comparison of sensitiveness with syphilitic serums with that of standard antigen, and correction of the antigen, if necessary, to the required degree of sensitiveness.

Uniformity of Standard Kahn Antigen.—Since the basis of comparison for the sensitiveness of new antigen is a standard antigen in use at the time, it is important to know whether the uniformity of antigen sensitiveness is constant from year to year. Accordingly, the following experiments were carried out.

Six lots of antigen were chosen from 15 lots: two were prepared during 1924, two during 1925, and two during 1926. One of these antigen (lot 14) was the one used in routine at the time of the experiment and was classed as the control. These six antigens were diluted with physiologic salt solution according to their respective titers and tested with ten serums (pooled) of varying potency. This was repeated three times until data with forty different serums were obtained. Table 6 summarizes the results of this experiment.

It is clear from this table that standard antigen is a product of marked uniformity. Although small variations in individual readings are found, the effect on the final result is negligible, due to the fact that the readings in the three tubes are averaged.

TABLE 5

ANTIGEN HAVING TITER OF 1 + 0.8 AND LESS SENSITIVE THAN STANDARD ANTIGEN CORRECTED BY 50% CONCENTRATION WITH ALCOHOLIC LIPOIDS

Serums	Antigen L														
	Antigen 5 Standard Antigen Titer 1 + 1.1			Unconcentrated Titer 1 + 0.8			25% Concentration Titer 1 + 1.1			50% Concentration Titer 1 + 1.1			75% Concentration Titer 1 + 1.1		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	+	++	+++	0	+	++	++	++	++	++	++	++	0	+	++
2	+	++	+++	0	0	++	++	++	++	++	++	++	0	+	++
3	0	++	+++	0	0	++	0	++	++	++	++	++	0	++	++
4	0	++	+++	0	0	++	0	++	++	++	++	++	0	++	++
5	0	++	+++	0	0	++	0	++	++	++	++	++	0	++	++
6	0	0	++	0	0	++	0	++	++	++	++	++	0	++	++

TABLE 6

COMPARATIVE SENSITIVENESS OF DIFFERENT LOTS OF KAHN STANDARD ANTIGEN PREPARED DURING 1924-1926

	Antigen 1 Titer 1 + 1 1/5/24	Antigen 3 Titer 1 + 1.1 11/1/24	Antigen 5 Titer 1 + 1.05 4/28/25	Antigen 7 Titer 1 + 1.05 8/7/25	Antigen 9 Titer 1 + 1.1 1/3/26	Antigen 14 Titer 1 + 1.1 11/1/26
Serums						
1-14	+	+	+	+	+	+
15	+	+	+	+	+	+
16	+	+	+	+	+	+
17	+	+	+	+	+	+
18-20	+	+	+	+	+	+
21	+	+	+	+	+	+
22	+	+	+	+	+	+
23	+	+	+	+	+	+
24	+	+	+	+	+	+
25	+	+	+	+	+	+
26	+	+	+	+	+	+
27-31	+	+	+	+	+	+
32-33	+	+	+	+	+	+
34	+	+	+	+	+	+
35-40	+	+	+	+	+	+

DISCUSSION

The fact that in the serum diagnosis of syphilis we are obliged to employ as antigens extracts of heart muscle, the chemical composition of which is little known, necessarily prevents the evolution of a test having the perfection of a chemical method. The Kahn test, however, has become established as a method possessing a high degree of reliability as a diagnostic agent. It seemed important therefore, to carry on studies on antigen which would extend our knowledge regarding its properties and thereby enhance the value of the test.

The well known lack of uniformity of antigen sensitiveness in complement fixation and precipitation tests for syphilis has led us first to investigate the problem of uniformity in connection with Kahn antigen. If antigen for the Kahn test, prepared in one laboratory, were to differ in sensitiveness from antigen prepared in another laboratory, then indeed, this test would be no improvement on older methods. The fact that we have succeeded during the past three years in employing an antigen of uniform sensitiveness, even though we have prepared during this period fourteen different lots of antigen of five to ten liters each, is to our mind significant, since it places the serum diagnosis of syphilis not far behind a chemical method. It is of interest that dilution of antigen with cholesterolized alcohol in some cases renders it more sensitive and in others less sensitive. According to unpublished data obtained in this laboratory, too high as well as too low concentration of antigenic lipoids tend to weaken precipitation reactions. If an antigen is not sufficiently sensitive due to excessive lipoids, dilution with alcohol will increase its sensitiveness. Then again, if an antigen is too sensitive, due conceivably to richness in a certain kind of lipoids, dilution with alcohol will reduce its sensitiveness. Additional experiments in connection with this problem will be presented elsewhere.

Turning to a practical phase of antigen uniformity, it is well to emphasize that the preparation and standardization of antigen for the Kahn test should be carried out in central laboratories where expert serologists are available for work of this nature. An antigen once standardized keeps at least three years and very likely indefinitely without further titration or standardization. We would recommend also, when obtaining a lot of powdered beef heart, to prepare a small amount of antigen as a trial to determine whether it produces a product which can be readily standardized. As soon as this is determined it is best to convert the beef heart into antigen, since the latter is not influenced by age, while the beef heart, especially if it contains moisture, may

undergo such changes in time as to render it unfit for use. Whenever it is necessary to keep powdered beef heart for any length of time, it is well to keep it at icebox temperature.

CONCLUSIONS

Different lots of beef heart produce Kohn antigen which may vary in sensitiveness. This is believed to be due, in part at least, to differences in the degree of autodigestion of beef heart, leading to varying lipoid concentrations of the antigen.

Antigen may be standardized to a high degree of uniformity in sensitiveness. An antigen too concentrated in beef heart extractives can be made standard by dilution with alcohol, and one not concentrated enough can be standardized by the addition of a titrated amount of extractives. The procedure for standardizing antigens of varying sensitiveness is given. Antigens prepared and standardized in these laboratories during 1924, 1925 and 1926 were found to be practically alike in sensitiveness.

FURTHER STUDIES ON STAPHYLOCOCCUS BACTERIOPHAGE

BESSIE R. CALLOW

From the Department of Bacteriology, College of Physicians and Surgeons Columbia University, New York

In 1922, I¹ reported studies on the phenomenon of d'Herelle with *Staphylococcus aureus* and called attention to the fact that a bacteriophage or lytic substance, specific for one or more strains of *staphylococcus*, can be isolated directly from acute *staphylococcus* boils. It was possible in only two cases of the ten reported to demonstrate direct action on the autogenous strain with a given lytic agent, thus calling attention to the apparent insusceptibility of the organisms directly involved to the lytic substance. These experiments, as also the work of most of the earlier writers, seemed to indicate that the initiation of bacteriophage activity is the result of contact of the organisms with body cells. However, the later work of Bail,² Otto and Munter,³ Kuttner⁴ and others who isolated bacteriophages from old broth cultures of organisms of the colon-typhoid group was of paramount importance because it suggested that the lytic principle might be derived from the organisms themselves without contact with body cells.

To multiply if possible the facts concerning the isolation of these materials from pure cultures this method of investigation was extended to *Staphylococcus aureus*. As a necessary introduction to the main part of this paper, I shall summarize briefly certain outstanding facts (heretofore unpublished) which developed in the early experiments.

A bacteriophage principle was isolated, transmissible in series, from broth cultures of ten of twelve strains. Filtrates of five strains, two of which were isolated several years ago, gave definitely regular results against their susceptible strains; the others gave positive but irregular results which often could not be duplicated. In no two cases were the filtrates active upon the same series of organisms nor was it possible to extend this selectivity to a greater number of strains. In many cases either the organism tested or the organism from which broth filtrates were obtained gave a history of previous contact with lytic material. However, this was not a fixed rule, there being instances of lytic activity and no history of previous lytic contact. Moreover,

Received for publication, Feb. 4, 1927.

¹ Callow: *J. Infect. Dis.*, 1922, 30, p. 6.

² *Wiener klin. Wchnschr.*, 1921, 34, p. ??

³ *Deutsche med. Wchnschr.*, 1921, 47, p. 52.

⁴ *J. Bact.*, 1923, 8, p. 1.

since isolation all strains showed only normal growths during repeated transplants on agar slants. The most active filtrates were obtained from young broth cultures ranging in age from six hours to one week, the younger filtrates being far more active than the older. Only a few weak filtrates were found after three weeks incubation.

A step considerably in advance of the broth culture method which developed from that research consisted in washing a lytic substance from growths on solid medium and subsequently passing these suspensions through a Berkefeld filter. In the preliminary experiments the following were used interchangeably as solvents: extract broth P_H 8.0; distilled water; 0.85% NaCl solution, and varying concentrations of NaOH in distilled water from .02 to .005% (M/200 to M/800). Of twelve strains treated in this manner, six gave positive results. These filtrates corresponded in activity with the broth culture filtrates for the same strains. All strains did not yield bacteriophage solutions of equal potency. By enormously increasing the number of organisms per given volume of solvent, it was possible to augment the activity of one from a titer of 1:10 to 1:500; of another 1:100 to as high as 1:1,000,000. In this manner active washings were obtained from three old strains which evidently produce phages of such low potency that they could not be identified when only a few organisms were washed.

It is of extreme interest to have isolated a bacteriophage in such a way that it is relatively pure in nature, that is free from all broth constituents which undoubtedly cause a modification of it so as to make a chemical analysis impossible. Detailed experiments on the washings of one selected strain follow. Whenever possible this "pure" phage will be compared with the broth phage, a bacteriophage obtained by means of the lysis of bacteria in broth. The experiments suggest, that the separation in this way of a bacteriophage from bacteria accompanying it in the natural state and unmodified by extraneous substances, results in its becoming more sensitive to certain chemical and physical changes.

One strain of staphylococcus was selected. Filtrates and washings of this organism had powerful bacteriophagic action on three other strains. The tests are confined to its action on one of these strains except in the few instances in which confirmatory tests were made simultaneously on one of the other strains.

For the purpose of the experiments large quantities of "pure" phage were prepared at one time, 200 to 300 cc. pyrex plates with tin covers, approximately 10 inches in diameter, were poured with extract agar (P_H 7.8) and seeded with a distilled water emulsion of staphylococci scraped from an 18 hours' growth on agar slants. After 18 hours incubation at 37.5 C., the growths were scraped from the surfaces into freshly distilled water, approximately 40 cc. to a plate, and the emulsion was left in the ice box for 48 hours. This was then centrifugalized at high speed for two hours and filtered through a Berkefeld candle. Preliminary titration against the susceptible strain was made by the dilution method. These solutions averaged a strength of 1:1,000,000, that is, such a dilution still caused the presence of lytic colonies in streaks made from the dilution tube but not generally a lysis of the whole culture with clearing. This clearing limit was reached at a dilution of 1:10,000. Subsequent stock solutions were adjusted to this titer, when necessary, to insure comparative results. Distilled water was selected as a solvent because it was the simplest and gave a satisfactory product. Care was always taken to use freshly distilled water (P_H 5.4-6.4) since the H-ion concentration of the solvent is a most important factor. The growths were scraped from the agar surfaces rather than washed off because by the latter method we would have extracted water soluble substances from the agar which

would have complicated the product and spoiled the results. The cocci were left in contact with the distilled water not longer than 48 hours because it was found that the longer the contact after that time the less active the resulting solution. This was due no doubt to a readsorption of the bacteriophage onto the cocci, possibly the dead ones, and a consequent permanent union.

The care of Berkefeld filters was important. I finally discarded the use of lysol for sterilization and of potassium permanganate and sodium bisulphide for cleaning as it was impossible to wash the filters free of these chemicals. I sterilized instead by boiling and washing with distilled water in large quantities, always reversing the filter and making sure that the last washing was neutral in reaction.

Glassware, such as stock bottles, test tubes and pipettes, were always left in acid water over night and then thoroughly washed in distilled water before sterilizing.

In making dilutions it was found necessary to use a new pipet for each dilution because the bacteriophage became easily absorbed to glass and was carried over beyond the amount needed. In a few early experiments this caused a serious error of several hundred points.

THE EFFECT OF CERTAIN NEUTRAL SALTS ON THE EXTRACTION OF BACTERIOPHAGE—IMPORTANCE OF HYDROGEN ION CONCENTRATION

Distilled water was found to be a satisfactory solvent with certain strains, but it was impossible to obtain bacteriophage from as many different strains or bacteriophage active upon as many different organisms as with old broth cultures. This may possibly have been due to factors in the broth such as H-ion or salt concentration or possibly aging of the cocci in the broth. I therefore modified the distilled water with different salts of varying strengths in an effort to secure a stronger bacteriophage.

Preliminary extractions were made with K_2HPO_4 , Na_2SO_4 , Na_2CO_3 , in M/1000 strengths and compared with similar extractions by double distilled water.

Large quantities of the cocci were grown on pyrex plates and the growths scraped into distilled water (40 cc. per plate). This suspension was divided into four equal parts. To the first, an equal quantity of distilled water was added, to the other three, equal quantities of M/500 solutions of the respective salts so that the final concentration of the salt equaled M/1000. These suspensions were placed in the ice box for 48 hours, when they were centrifugalized and filtered. The filtrates were compared by the dilution method with six or more strains of staphylococcus. The controls consisted of one for the susceptible organisms without bacteriophage and another one for each of the salts used in the same relative concentration.

I found that the extract with K_2HPO_4 was 200 times as strong as the distilled water extract. The Na_2SO_4 extract was 20 times as strong and the Na_2CO_3 twice as strong. The phosphate and sulphate extracts were also active on two other strains upon which the distilled water extract was inactive, and phosphate extracts of other organisms showed this increased selectivity. The

phosphate extracts were always the most powerful, some being 1000 times as strong as the distilled water extracts. Similarly when K_2HPO_4 and Na_2SO_4 were added directly to the distilled water extract to give a final concentration of M/1000, an increased amount of phage action was found but this was less striking than when the organisms themselves were extracted with the salts.

The next experiment dealt with the effect of extracting the active strain with both acid and basic phosphates of sodium and potassium (M/1000) and comparing the extracts with a distilled water extract. The results with the acid and basic phosphates were comparable, and the change from the sodium cation to potassium cation caused no appreciable difference. The acid phosphate extracts, however, were not as powerful as the distilled water extract, but the basic phosphate extracts were roughly a thousand times as strong. The experiment suggested therefore that alkalinity was the activating factor to be considered rather than the salts.

As previously mentioned the water used for extraction was doubly distilled. Its H-ion concentration under these conditions was therefore below P_H 7.0, averaging P_H 5.4-6.4. A M/1000 solution of basic phosphate (M or K) was found to be about P_H 8.6; the acid phosphates P_H 4.6; Na_2SO_4 , P_H 6.8 and Na_2CO_3 P_H 9.6. I therefore compared extracts with K_2HPO_4 (M/1000), distilled water at P_H 5.4 and distilled water brought to the same P_H as the phosphate solution with NaOH (about P_H 8.4). As before, the phosphate extract was 1000 times as strong as the one made with distilled water at P_H 5.4 but was only 10 times as strong as the extract with distilled water adjusted to a similar alkalinity.

It seemed, therefore, that the increased effect of the addition of alkaline phosphates in low concentrations to the solvent was not necessarily an activating effect on the part of the salt itself, but simply that it produced a more favorable alkalinity. In other words, these experiments show that the "pure" phage of this staphylococcus is without question extremely sensitive to changes in H-ion concentration; a change from P_H 5.4 to P_H 8.0-8.4 making a tremendous difference in its lytic power. Also that an alkalinity of P_H 8.0-8.4 is the most favorable, since extracts made with dilute Na_2CO_3 at P_H 9.6 showed a decrease in the other direction. Similarly when samples of distilled water phage were adjusted to a series of different H-ion concentrations from P_H 2.4-9.4 with sterile HCl and NaOH, it was found that destruction was complete below P_H 4.4 and greatly diminished at P_H 9.4. These limits are comparable to the P_H values of double distilled water and Na_2CO_3 M/1000. This showed in another way that the pure distilled water bacteriophage falls within the same limits of acidity and alkalinity heretofore ascribed to all broth bacteriophage.

THE RELATION OF BACTERIOPHAGE ACTIVITY TO THE CONCENTRATION OF THE SALT USED IN THE EXTRACTION

It was previously determined that salts such as K_2HPO_4 and NaCl used in concentrations greater than M/1000 were decidedly toxic to the distilled water bacteriophage. It seemed necessary to find the limits of this toxicity for a number of salts, choosing for the experiment K_2HPO_4 and two series of salts, namely: NaCl, Na_2SO_4 , Na_3 citrate, $Na_4Fe(CN)_6$ and NaCl, $CaCl_2$, $LaCl_3$, using the distilled water extract as a control. The salts were used in concentrations ranging from M/10 to M/10,000. The work of Bronfenbrenner and Korb⁵ with salts and broth bacteriophage is of interest in this connection.

In order to eliminate the factor of H-ion concentration, all salt dilutions and distilled water were adjusted to the same P_H (7.8 to 8.0) with NaOH or HCl. It was found that most of the solutions, particularly the distilled water, changed rapidly after adjustment, consequently it was necessary to add the organisms immediately. Each suspension was checked, colorimetrically, before filtering and, if necessary, after filtering it was readjusted to that reading. The filtrates were always slightly more acid than the original solution, averaging about P_H 7.4-7.6, this being true not only of the different salts but of the various dilutions of all of them. However, the H-ion concentration thus obtained remained constant indefinitely.

As it was necessary for comparison to expose approximately the same number of cocci to all of the salt dilutions and since the experiments could not be done all at the same time, a series of $BaSO_4$ standards were prepared according to the method of Kolmer. The one selected corresponded to 250 million staphylococci per cc. and in each experiment the stock suspension of cocci was adjusted to this standard before dilution with the salt. To further check the results for the final comparison, the number of cocci in the stock suspensions was often counted by the dilution agar plate method. Also to discover the direct effect of the salt, a final count was made of each suspension just before filtering.

Series I.—Extractions of the staphylococcus were made with distilled water and a series of dilutions of the following salts giving a final salt concentration of M/10, M/100, M/1,000, M/10,000: K_2HPO_4 , NaCl, Na_2SO_4 , Na_3 citrate, $Na_4Fe(CN)_6$, $CaCl_2$, $LaCl_3$. With the exception of $CaCl_2$ and $LaCl_3$, dilutions of M/1000 or higher were nontoxic; the strength of these filtrates was com-

⁵ J. Exper. Med., 1926, 43, p. 71.

parable to the distilled water extract. Dilutions of M/100 showed a varying degree of toxicity; M/10 dilutions of all the salts was almost completely destructive (table 1).

Series 2.—The effects of anions of increasing valences was tested. In series A extractions were made in parallel with M/100 solutions of each salt, using

TABLE 1

ACTIVITY OF STAPHYLOCOCCUS BACTERIOPHAGE IN DIFFERENT CONCENTRATIONS OF K_2HPO_4

Extract Dilution	Concentration of Salts Used in Extraction				
	M/10	M/100	M/1000	M/10,000	H ₂ O
Concentrated.....	++	++++	++++	++++	++++
1:10.....	0	++++	++++	++++	++++
1:100.....	..	+++	++++	++++	++++
1:1000.....	..	+	++	++	++
1:10,000.....	..	0	+	+	+
Salt control.....	0	0	0	0	0
Staphylococcus control.....	0	0	0	0	0

Table 1 is illustrative of all the results in series 1 except those with $CaCl_2$ and $LaCl_3$.

++++ = active bacteriophage action; 0 = no bacteriophage action, in all tables.

TABLE 2

ACTIVITY OF BACTERIOPHAGE IN PRESENCE OF ANIONS OF INCREASING VALENCE

Highest Dilution of Extract Showing Bacteriophage Activity
(+ to +++)

Salts in Final M/1000 Concentration

	NaCl	Na ₂ SO ₄	Na ₃ C ₆ H ₅ O ₇	Na ₄ Fe(CN) ₆	H ₂ O Distilled
A.....	10,000 ++++	100 +++	10 +++	10 +	10,000 ++++
B.....	10 ++++	Concentrated 0	Concentrated 0	Concentrated ++	1,000 ++++

Control of each salt (M/100) + Staphylococci : 0.

Control of staphylococci minus salts and extract : 0.

TABLE 3

ACTIVITY OF BACTERIOPHAGE IN PRESENCE OF CATIONS OF INCREASING VALENCE

Highest Dilution of Extract Showing Bacteriophage Activity
(+ to +++)

	NaCl	CaCl ₂	LaCl ₃	H ₂ O Distilled
A. M/100.....	1000 ++	.. 0	.. 0	100,000 ++++
A. M/1000.....	100,000 +	1000 ++++	.. 0	100,000 ++++
B. M/1000.....	100 ++++	Concentrated +	.. 0	10,000 ++

Control of each salt (M/100, M/1000) + staphylococci : 0.

Control of staphylococci minus salts and extract : 0.

the same stock suspension of organisms and all were tested after 48 hours under like conditions and in series B the distilled water filtrate from series 2-A was divided into five parts, diluted 1:1 with M/50 concentrations of each of the salts and tested after five days.

Series 3.—The effect of cations with increasing valence was also studied. In series A extractions were made in parallel with M/100 and M/1000 solutions of each salt using the same stock suspension of organisms and all were tested after 48 hours under like conditions. In series B, distilled water filtrate from series A was divided into four parts, diluted 1:1 with M/500 concentrations of each of the salts and tested after five days.

These experiments show that the concentration of the neutral salt is an additional factor in the modification of bacteriophage activity. By adjusting all salt dilutions to the same P_H the H-ion factor was eliminated. Concentrations of individual salts were ineffective above M/1000 dilutions but concentrations of M/100 or less were destructive. Comparison of a series of salts (M/100) in which the anion was changed showed a decreased activity as the valency was increased. A variation in the cation as in the NaCl, CaCl_2 , LaCl_3 series showed a similar effect. The inactivation corresponded to the change in valency of the anion or cation, and to the concentration of the salt. These salts not only produced their effect upon the extraction of bacteriophage from the cocci but also upon the bacteriophage after extraction with distilled water, as is shown under B in tables 2 and 3.

It is striking, however, that the change in concentration of the anion had a greater effect than varying the anion itself since M/100 solutions of both CaCl_2 and LaCl_3 were decidedly destructive as compared with M/100 NaCl whereas M/100 Na_2SO_4 , $\text{Na}_3\text{citrate}$ and $\text{Na}_4\text{Fe}(\text{Cn})_6$ were less so. These salts not only effected the extraction of bacteriophage from the organisms but also to a far greater degree the bacteriophage after extraction with distilled water. In the latter case the salts were probably acting in their full concentration. With organisms present it is conceivable that at least part of the ions were removed from solution by union with amphoteric protein substances of the bacteria, so that a relatively less amount of salt was available for action on the bacteriophage. Counts of the organisms were made in most of these experiments before addition of the salt and later before filtering. It was impossible to secure consistently accurate counts but in all suspensions at least one third of the organisms were destroyed within 4 to 5 days; with the exception of the most dilute solutions of NaCl and phosphates the distilled water destroyed the fewer number of organisms; and the destruction ran in parallel with the increase in valence of the anion or cation and was proportional to the concentration of the salt.

It is impossible to discount the fact that this destruction of the organisms might in part account for the increasingly smaller proportion

of principle extracted. However, we know that salts added directly to a distilled water extract with no cocci present have an even greater toxic effect on the principle. That the principle is extraordinarily sensitive to all modifying factors was shown also by extracting equal quantities of organisms with double distilled water and tap water, the former being adjusted to P_H 7.0 of the latter. The principle obtained with distilled water was at least ten times more active. It is noteworthy that the tap water contains a large amount of calcium.

It is evident therefore that in the extraction of bacteriophage substances from this staphylococcus the most active and powerful principle was obtained with distilled water adjusted to P_H 8.0 with alkaline phosphates (M/1000) or NaOH and that the addition of any one of a series of salts beyond a certain concentration was decidedly deleterious to the principle.

THE EFFECT OF REPEATED EXTRACTION OF THE SAME ORGANISMS
WITH DISTILLED WATER. OTHER ATTEMPTS TO
OBTAIN BACTERIOPHAGE

It was impossible to extract all of the bacteriophage principle from a suspension of the staphylococcus with one exposure to distilled water or even with several successive ones. A decreasing amount of principle was present in each filtrate but no one of six or seven was entirely free from it. In such experiments it was possible to extract greater amounts of bacteriophage at one time with a small than with a large amount of solvent. An indefinite exposure of a week or longer as compared with one made within three days, produced a much less active principle. Either the amount that was originally thrown out into the medium was readsorbed, or toxic substances were given out which destroyed some of the principle.

Several unsuccessful attempts were made to extract lytic substances with distilled water and phosphate solution from dead organisms which had been killed with heat or ether, alcohol and other disinfectants. Even grinding the bacterial powder in a small Abbé mill, in an effort to rupture the cell membrane, was of no avail.

THE EFFECT OF HEAT ON THE DISTILLED WATER BACTERIOPHAGE
AS COMPARED WITH THE BROTH BACTERIOPHAGE

An exposure of one half hour at 60 C. was sufficient to destroy two bacteriophages of like titer (1:100,000) at P_H 7.4. It made no difference whether the fluid was heated within sealed or open capsules. It

is conceivable that a modification of this experiment could be made by changing the H-ion concentration of the solutions before heating. The broth bacteriophage used here and in the next experiment was made by the lysis in broth of one of the susceptible strain by an active broth culture filtrate of the active strain. This filtrate was carried in series until the desired titer was obtained.

THE EFFECT OF ALCOHOL, ACETONE, TOLUOL, TRICRESOL,
CHLOROFORM AND ETHER

It was impossible to precipitate bacteriophage substances from distilled water extracts of the staphylococcus (titer 1:100 million) with any % of absolute alcohol or acetone. No precipitate was found in any of the tubes even on standing three days in the ice box. Evaporation of the alcohol and acetone after a short exposure revealed that the reagents were rapidly destructive in all proportions. Dilutions of the distilled water bacteriophage with equal quantities of broth did not result in the preprecipitation of a lytic principle.

On the contrary I could without difficulty precipitate lytic substances from the broth bacteriophage with both alcohol and acetone using the reagents in the proportion of 2 to 1. These results corroborated in general the observations of others, notably Kabeshima,⁶ d'Herelle⁷ and Bronfenbrenner and Korb.⁸ I agree with Bronfenbrenner and Korb that the destruction is rapid, results in the loss of most of the principle within a few minutes, and takes place more rapidly at room temperature than in the ice box. The bacteriophage must be present therefore in certain definite amounts, otherwise because of the rapid destruction a dilute phage would not be demonstrable in the precipitate. Acetone proved to be the more satisfactory precipitating agent, being not only less destructive than the alcohol but it also would precipitate the active principle out of a dilute solution (1:100) whereas the alcohol failed to do so (see table 4).

A distinct difference was therefore shown in the two bacteriophage solutions of the same titer. The one containing no accessory broth constituents and free so far as can be judged from the disintegration products of bacterial lysis is non precipitable. The other formed as the result of lysis of organisms in a broth medium is readily precipitable by alcohol and acetone. It would seem that in the latter the bacterio-

⁶ Compt. rend. Soc. d. biol., 1920, 86, p. 360.

⁷ Le Bacteriophage-son Rôle dans l'immunité, 1921; Immunity in Natural & Infectious Diseases (Translation), 1924.

⁸ Soc. Exper. Biol. & Med., 1924, 21, p. 4.

phage is not precipitated directly, but mechanically carried down by the precipitated broth proteins. The fact it is not completely destroyed at once is probably due to protective colloids surrounding it. However, the fact that the distilled water extract when diluted with broth fails to yield active substances in precipitation would suggest that the bacteriophage has an intimate relation to the substances in the broth.

In order to show if an unfiltered distilled water extract would be more powerful than a Berkefeld filtrate of the same extract it was necessary to obtain a sterile product. A sufficient amount of heat to destroy the cocci caused a loss of most of the principle. For a similar reason toluol and tricresol were discarded, the necessary 0.5% was just sufficient to sterilize and caused only a small reduction in the activity of the bacteriophage. When chloroform was added the tubes were immediately corked to avoid evaporation. After 24 hours in the ice box

TABLE 4
COMPARISON OF ALCOHOL AND ACETONE PRECIPITATION OF BROTH BACTERIOPHAGE

	Alcohol Precipitation		Acetone Precipitation	
	(Bacteriophage)		(Bacteriophage)	
	Concentrated	1:100	Concentrated	1:100
3 hours.....	++++	0	++++	+++
24 hours.....	++++	0	++++	+++
48 hours.....	++++	0	++++	++
Bacteriophage control.....	++++	++++	++++	++++
Staphylococcus control.....	0	0	0	0

++++ = active bacteriophage action; 0 = no bacteriophage action.

chloroform was removed by evaporation in the incubator at 37.5 C. A number of tests were made in parallel with filtered extracts—in some the two were equal in strength, in others there was a reduction in activity from 10 to 100 times.

Ether had no injurious effect on the distilled water extract.

DIALYSIS

Collodion sacs were made as described by Eggarth.⁹ A graded series was obtained by varying the amounts of alcohol and ether in the solvent, the higher the proportion of alcohol the more permeable the membrane. The sacs were sterilized in the autoclave for ten minutes at 10 pounds pressure. Five cc. of the test substance was used and the sac containing this immersed in 15 cc. of sterile distilled water adjusted to P_H 7.0. The tests were always made in duplicate, using four sets of bags at a time, each with a different permeability. Dialysis was

⁹ Biol. Chem., 1921, 48, p. 1.

carried on in the ice box at 4 C. Samples taken after 2 hours, 24 hours, and 48 hours were tested against the susceptible strain of staphylococcus in the usual manner.

It was found in all the experiments that membranes made with a 40-60 ether-alcohol solvent were in the majority of the tests impermeable to the bacteriophage. Sacs made less permeable still, namely, with a 50-50 mixture were always impermeable. However, membranes of greater permeability, that is, those made with a percentage of alcohol higher than 60% allowed rapid diffusion of the principle from the sac.

Attention is called to the work of d'Herelle⁷ and Marshall¹⁰ who have made dialysis experiments with broth bacteriophages.

ADSORPTION AND ELUSION

It has been known for several years that bacteriophage can be adsorbed out of a solution. Jaumin and Meuleman¹¹ showed again that the principle is adsorbed by dead bacteria specifically. Studies on the adsorption of colloids has been promoted through the work of De Porter and Maison¹² who worked with animal charcoal and De Necker¹³ who used a large series of metal colloids, particularly $\text{Al}(\text{OH})_3$. With this DeNecker claims to have been able not only to adsorb the principle out of solution but also to recover it again by acidifying with dilute acetic acid until the precipitate was dissolved. Of particular interest is the recent work of Willstätter on the adsorption and elusion of a number of enzymes in an attempt to purify them. I tried similar experiments with the distilled water bacteriophage with some success. Gildemeister and Hersberg¹⁴ experimented on the adsorption of bacteriophage with infusorial earth in acid medium and its recovery again by alkalinizing with ammonium hydroxide.

Kaolin Experiments.—It was necessary to thoroughly clean the commercial kaolin before using it. This was done by several hours boiling in hydrochloric acid followed by repeated washings with water in order to free the kaolin of all traces of acid. Kaolin treated in this way was more easily handled, absorbed more readily and only half the amount was necessary for a given solution. Using a stock solution with a titer of 1:100,000, 0.2 grams of washed kaolin completely adsorbed 2.5 cc. of the solution, whereas 0.4 grams of stock kaolin were necessary.

It is apparent that a certain definite amount of kaolin is necessary to completely adsorb all of the principle from a given solution. Kaolin readily settles

¹⁰ J. Infect. Dis., 1925, 37, p. 126.

¹¹ Compt. rend. Soc. de biol., 1922, ??, p. 362.

¹² Arch. Internat. d. Pharmacod., 1921, 25, p. ??.

¹³ Compt. rend. Soc. d. biol., 1920, 87, p. 1247.

¹⁴ Centralbl. f. Bakteriol., 1, O., 1924, 91, p. 228.

out so that by shaking the suspensions it was possible to facilitate adsorption by 38%. The adsorption also varied with the H-ion concentration of the solution, a medium of P_H 8.4 producing better extraction than one of P_H 6.8.

Adsorption with Alumina Cream.—The aluminium hydroxide was made by adding 5 cc. NH_4OH (10%) to 50 cc. $Al_2(SO_4)$ (5%). This mixture was filtered and the precipitate washed several times with distilled water until the suspension was neutral in reaction. It was found unsatisfactory to sterilize by autoclaving at ten pounds pressure for ten minutes because the H-ion concentration of the cream was increased to P_H 6.0-6.2. However, boiling ten minutes over the free flame was sufficient to sterilize and the acidity was not altered below two points.

To test the adsorption powers varying amounts of the cream (from 5 cc. to 0.5 cc.) were centrifugalized at high speed and the supernatant fluids discarded. Five cc. of a stock distilled water extract was added to each sediment titer 1:100,000). The tubes were intermittently shaken and left in the ice box for 48 hours. They were again centrifugalized and the supernatant fluids tested for the presence of lytic principle. It was found that 2.5 cc. of the stock aluminum hydroxide completely adsorbed all the lytic principle from 5 cc. of distilled water extract. The same quantitative relationship existed therefore between the gel and lytic principle as in the experiment with kaolin.

Elution Experiments.—At various times it was possible to successfully elude the bacteriophage in small amounts from kaolin with M/250 and M/500 solutions of three salts, namely K_2HPO_4 , Na_2SO_4 and Na_2CO_3 . At times the results were strikingly positive, particularly with solutions of M/500 strength. At other times successful results were obtained with one salt and not with another. In no case was an amount recovered that was proportionate to that absorbed. Positive results were not obtained when more concentrated solutions of the same salts were used. Phosphate buffer solutions giving a range in P_H from 5.8 to 8.0 were diluted so that the concentration of K_2HPO_4 present was not greater than M/250. Lytic substances were successfully eluded from the kaolin with three of these mixtures, namely solutions corresponding to P_H 7.0, 7.8, 8.0.

I was unsuccessful in recovering the slightest trace of lytic principle from the aluminum hydroxide. I used all the neutral salts mentioned in the preceding experiments in various concentrations; also phosphate buffer solutions, and several carbohydrates. I also tried dissolving the hydroxide with dilute acetic acid as DeNecker had done. This too was unsuccessful. Even if the principle had been freed by the acid the change in H-ion concentration should have been sufficient to destroy it.

PROTEIN COLOR TESTS

The distilled water extract containing bacteriophage does not respond to any of the protein color tests (Millon's, Hopkins-Cole, xanthoproteic or biuret) nor is it precipitated by Tonnet's reagent. However, this does not entirely eliminate the possibility of its protein nature, since it may be present in a dilution that is too high to give such tests. The solutions tested, however, were of high titer from a bacteriophage point

of view, namely 1:100,000 to 1:1,000,000. So far it has been impossible to devise a means of concentrating large quantities of the solutions without injury to the lytic property. It is impossible, therefore, at the present time to thoroughly investigate its chemical nature.

SUMMARY

By passing a staphylococcus suspension in distilled water through Berkefeld filter, a bacteriophage was obtained that was free from broth constituents and did not give any protein color tests. Adding any one of several salts in concentration greater than M/100 proved injurious to the bacteriophage, the activity of which decreased as the valence of the anion or cation of the salt increased. Changing the H-ion of the distilled water from P_H 5.4 to 8.0 increased the strength of the bacteriophage several hundred times. Bacteriophage was not precipitated by absolute alcohol or acetone. Heat at 60 C. for half an hour and various chemicals destroyed the bacteriophage. Chloroform (0.6%) sterilized the water without injuring the bacteriophage, but toluene and tricresol (0.5%) were injurious, while ether had no effect. Kaolin and alumina cream adsorbed the bacteriophage; under certain circumstances, small amounts could be eluded from the kaolin only. Collodion sacs made with ether (40 parts) and alcohol (60 parts) withheld the bacteriophage while more permeable sacs allowed rapid diffusion.

THE EFFECTS OF FORMALDEHYDE ON SMOOTH MUSCLE CONTRACTION IN ANAPHYLAXIS

STUDIES IN BACTERIAL METABOLISM LXXXIV

ARTHUR ISAAC KENDALL, HARRY L. ALEXANDER
AND
JANET A. HOLMES

*From the Departments of Medicine, and of Bacteriology and Public Health, Washington
University School of Medicine, St. Louis*

It was shown that formaldehyde¹ will promptly relax a contraction induced in a strip of smooth muscle by histamine or the histamine-like substance produced by some strains of the gas bacillus.² Formaldehyde will also prevent such contractions from taking place. The unmistakable similarities between histamine reactions and those induced by anaphylaxis, commented on by many observers,³ suggest very naturally that formaldehyde may also influence the anaphylactically induced contraction in smooth muscle, if there be, indeed, a chemical relationship between the two. It is quite obvious, of course, that a favorable outcome of a series of experiments designed to test this possibility would not prove histamine to be the long sought for substance that specifically causes smooth muscle to contract in anaphylaxis; nevertheless, it should shorten somewhat the approach to this puzzling problem.

Methods.—Guinea-pigs weighing approximately 250 gm. were actively sensitized to fresh egg albumen by intraperitoneal injections of 0.2 cc. of a 1:10 solution each, or passively sensitized by injection with 0.2 cc. of a potent, anti-egg white rabbit serum. In the former, 14 days usually elapsed between the sensitizing injection and the test: in the latter, the experiment was made 24 hours after the injection of the specific serum. Virgin uterus, and strips of small intestine were used throughout the investigation as the reactive smooth muscle. The animal was killed by a blow upon the head. The organs were either used immediately, or stored for brief periods in the icebox, in shallow, covered dishes moistened with Tyrode solution (P_H 7.9 to 8.0). The muscle preparations at the proper time were suspended in baths holding 150 cc. of Tyrode solution, and two of these Tyrode baths were suspended,

Received for publication, May 3, 1927.

¹ Kendall: Proc. Soc. Exper. Biol. & Med., 1927, 24, p. 316.

² Kendall and Schmitt: J. Infect. Dis., 1926, 39, p. 250.

³ Dale and Laidlaw: J. Physiol., 1911, 23, p. 182. Vaughan: Protein Split Products, 1913.

side by side, in an electrically heated water jacket maintained at a uniform temperature of 37 C. Oxygen was bubbled through each bath continuously. Each pair of Tyrode baths was exposed thus to the same physical conditions.

Changes in length of each muscle were recorded in the usual manner by means of a light lever writing upon a smoked drum. The ratio of lever arms was 1 to 8. Shortening of the muscle was indicated by an upward movement of the lever: relaxation by a downward movement. The time intervals were recorded simultaneously as part of the record.

Most of the experiments were performed in the following manner: two horns of the same sensitized uterus, or two successive, equal strips of sensitized intestine were suspended, each in its separate Tyrode bath. One of these strips of muscle served as a control. The other was the test strip. All controllable conditions were the same up to the moment of the experiment.

Expcr. 1.—Occasionally, both uterine and intestinal strips from a sensitized animal were observed to be spontaneously contracted. Formaldehyde up to a final concentration of 1:750, (0.2 cc. neutral formalin to 150 cc. Tyrode solution), was added to these. The formaldehyde had no appreciable effect. It should be emphasized that comparatively few of these spontaneously contracted strips were met with. In all 6 were encountered. Figure 1 is a reproduction of a kymograph record of one of these, to which 1 cc. of 1:10 dilution of formalin had been added, as indicated above.

Expcr. 2.—Strips of sensitized smooth muscle that had been exposed to neutral formalin solution (0.1 cc. in bath that held 150 cc.) were washed thoroughly with fresh, warm Tyrode solution, and then tested for contractability with histamine solution. Characteristic shortening of the muscle occurred in 14 of these; a few, 2 in number, failed to contract, however. Formaldehyde in the concentration used therefore does not injure smooth muscle to an appreciable degree. Figure 2 shows one of these histamine contractions, obtained after the above treatment with formaldehyde.

Expcr. 3.—Two horns of the same sensitized uterus, or two equal strips of the same sensitized intestine were suspended each in its Tyrode bath as indicated above. Formaldehyde (0.1 cc. formalin solution) was added to one bath, the other remaining a control. Shortly afterwards, egg white was added equally and simultaneously to each of the two

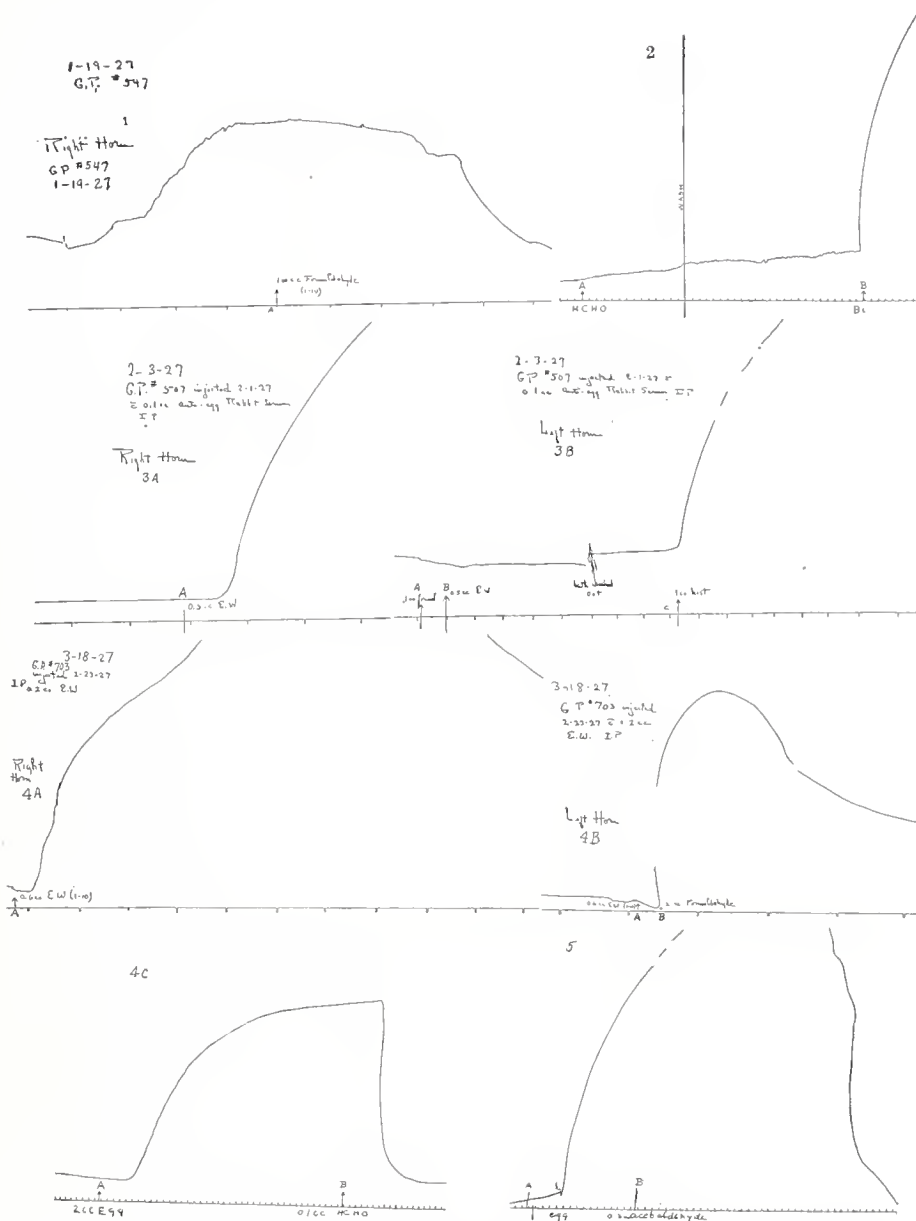


Fig. 1.—Spontaneously contracted guinea-pig uterus. 1 cc. formaldehyde solution * added at A. No effect. Time interval, 1 minute.

Fig. 2.—1 cc. formaldehyde added to uterine horn at A. No effect. Horn washed with fresh, warm Tyrode solution. 0.1 cc. histamine solution added at B. Prompt contraction induced.

Fig. 3A.—Right horn. Control. 0.5 cc. egg white (1:10 solution) added to Tyrode bath at A. Typical anaphylactic contraction induced. Fig. 3B.—Left horn. 1 cc. formaldehyde solution added to Tyrode bath at A. No effect. 0.5 cc. egg white (1:10 solution) added at B. No contraction. Uterine strip washed with Tyrode solution. At C histamine solution added. Typical contraction induced. Time interval, 1 minute.

Fig. 4A.—Control. Egg white added to sensitized horn of uterus at A. Typical, sustained contraction induced. Time interval, 1 minute. Fig. 4B.—Egg white added to sensitized horn of uterus at A. Contraction induced; then 2 cc. formaldehyde solution added at B. Contraction released. Time interval, 1 minute. Fig. 4C.—Egg white (2 cc. of 1:4 solution) added to strip of small intestine from a sensitized guinea-pig at A. Typical anaphylactic contraction induced. At B. 1 cc. formaldehyde solution added. Prompt relaxation. Time interval, 3 seconds.

Fig. 5.—Sensitized strip of small intestine from guinea-pig. At A. egg white I 2 cc. of 1:4 solution I added. Contraction induced. At B. 0.3 cc. acetaldehyde added. Contraction released. Time interval, 1 second. Control curve, showing prolonged contraction omitted.

* The formaldehyde solution used throughout these experiments was a 1:10 dilution of formalin.

baths. The strip untreated with formaldehyde usually contracted in the characteristic manner (fig. 3 A). This shows of course that the muscle was sensitized.

Sometimes the muscle failed to contract in response to the egg white. It was assumed in such cases that the animal was not sensitized and the experiment was rejected.

In most instances the control muscle contracted in response to the egg white and the other muscle strip which had been treated with formaldehyde, either failed to contract (fig. 3 B), or shortened by a small amount. Thus, in a series of 28 guinea-pigs, from each of which the control muscles were shown to be sensitized, formaldehyde reacted with the strips as follows: in 20, contraction was prevented completely; in 5, slight contraction occurred; in 3, formaldehyde had no appreciable effect. The muscle strips exposed to formaldehyde were tested subsequently as in experiment 2. Provided the formaldehyde in the bath did not exceed materially the equivalent of 0.1 cc. formalin solution, these strips usually contracted characteristically. It is worthy of note that the gut reacts rather more sluggishly to the antigen than the uterus, and strips of intestine from passively sensitized guinea-pigs have been found less satisfactory upon the whole than those from actively sensitized animals.

Expt. 4.—Two horns from the same sensitized uterus, or two consecutive, equal sensitized strips of small intestine were thrown simultaneously into contraction with equal amounts of egg white. If they failed to contract, the animal was not properly sensitized, and the experiment was discarded. When the two muscle strips contracted simultaneously, one served as a control (fig. 4 A). Formaldehyde in the proper concentration was added to the other which usually relaxed promptly (fig. 4 B). Figure 4 C, shows the same effect induced in an intestinal strip. The control tracing is omitted.

It was of course essential that the untreated muscle remain shortened during the period of this experiment.

In many instances, especially when the control contraction was maximal, formaldehyde failed to induce relaxation. No explanation can be proffered at this time to account for this failure of formaldehyde to relax such muscles.

This experiment was repeated 47 times. Formaldehyde relaxed 34 of these strips and failed to relax 13.

Exper. 5.—Sensitized uterine or intestinal strips, treated with formaldehyde as in experiment 3 were washed thoroughly with warm Tyrode solution to remove both the formaldehyde and the egg white. It was found then that such a muscle would not respond by contracting upon a subsequent introduction of egg white. This experiment was repeated 18 times. In no instance did the second trial with egg white cause a contraction. The muscle would, however, usually contract characteristically in response to a subsequent histamine stimulus. Contractions were obtained thus with histamine in 5 out of 7 strips. It is worthy of note that this histamine-induced contraction relaxed promptly in response to formaldehyde.

The muscle appeared to have been desensitized by the formaldehyde-egg white treatment, but it still usually possessed its power of contracting to histamine stimulation.

DISCUSSION

There are two outstanding features in these experiments. On the one hand there is exquisite specificity of the sensitized tissue to the homologous protein. This has long been recognized. On the other hand, in striking contrast to this specificity, formaldehyde relaxes anaphylactic contractions induced not only by egg white, but by other antigens as well, inasmuch as similar results have been obtained with horse dander and horse serum.

It should be stated at this point that certain aldehydes other than formaldehyde have a similar relaxing power for anaphylactically induced contractions (fig. 5).

Finally, there is evidence that formaldehyde will relax contractions that have been induced in smooth muscle by other chemical means, as by histamine. This leads to a consideration of the two prevailing theories of anaphylactic reaction.

One theory postulates the anaphylactic reaction as colloidal changes within the muscle. The other theory predicates a chemical reaction between the sensitized smooth muscle and the homologous protein, in which histamine, or a histamine-like substance is a significant factor. The similarities between histamine contractions and anaphylactic contractions in smooth muscle, and especially their common relaxation to formaldehyde, and certain other aldehydes, would seem to afford some support to the chemical theory. This does not however, constitute proof.

As a matter of fact, satisfactory evidence will be difficult to procure. Not only are the reagents, especially the histamine-like substances, reac-

tive in extremely small amounts, but also, the formaldehyde-histamine and formaldehyde-anaphylactic reactions discussed above apparently take place within the muscle, where conditions may be quite different from those in a test tube.

SUMMARY

The observations presented herewith seem to demonstrate three distinct, but related facts: formaldehyde, in suitable, small concentrations prevents a sensitized smooth muscle from contracting on contact with its homologous antigen; formaldehyde, in suitable, small concentrations relaxes anaphylactic contractions induced in sensitized smooth muscle; and, a strip of sensitized muscle exposed under proper conditions to formaldehyde and then to the homologous antigen, will not respond with a contraction on subsequent contact with the homologous antigen. This would seem to indicate that the muscle has been desensitized.

THE PHYSIOLOGIC ACTION OF HISTAMINE APPLIED DIRECTLY TO THE MUCOSA OF THE ISOLATED SURVIVING INTESTINE OF THE GUINEA-PIG

STUDIES IN BACTERIAL METABOLISM. LXXXV.

ARTHUR ISAAC KENDALL AND PHILIP LEONARD VARNEY

*From the Department of Bacteriology and Public Health, Washington University,
School of Medicine, St. Louis, Mo.*

Histamine, a very important biogenous amine produced in varying amounts by certain bacteria under quite definite conditions from the normal amino acid, histidine, induces powerful contractions in isolated, surviving smooth muscle. This has long been known. It is said to have little or no effect on skeletal muscle and at most but a very moderate effect on heart muscle.

Histamine is reactive physiologically in extremely small concentrations. Guggenheim¹ states that even as little as one part of histamine dissolved in 500-million parts of a physiologic solution may induce a minimal response in an isolated, surviving strip of small intestine from a guinea-pig. It is also well established that a small amount of histamine will cause a rapid and considerable fall in the blood pressure of an animal if it be injected quickly into the circulation. If on the contrary, the injection rate be slow, the blood pressure change may be slight or even not detectable.

Quite in contrast to these unquestioned effects of histamine on blood pressure and on the isolated, surviving intestine, is the seeming inertness of this substance when it is applied directly to the mucosal surface of the intestine. The more recent experiments of Meakins and Harrington,² and of Koessler and Hanke³ who have introduced large amounts of histamine dichloride into the alimentary canals of animals, do not seem to have resulted in the detection of any significant physiologic response which could be attributed to the effect of the substance itself. However, Koessler and Hanke state that when relatively large amounts of histamine dichloride are introduced in a capsule into the stomach of a guinea-pig, some of the amine may be identified later in the tissues of the alimentary canal, and some in the substance of the liver. In one such experiment the animal showed signs of discomfort during the first hour after the ingestion of the drug. There was depression, occasional sneezing, and the animal scratched its nose and was salivated. These symptoms soon wore off, however. The opinion

Received for publication, May 7, 1927.

¹ Die Biogenen Amine, 1924, p. 219.

² J. Pharm. & Exper. Therap., 1921, 18, p. 455; 1922, 20, p. 45.

³ J. Biol. Chem., 1924, 59, p. 889.

is expressed by them that some histamine may be absorbed through the enteric mucosa, it being rendered inert during its passage through the intestinal wall.⁴

It must be realized, also that the identification of imidazoleacrylic and beta imidazole acetic acids in urine,⁵ as well as the depressor substances⁶ found in some urines⁷ and in normal blood serum,⁸ points to at least some absorption of histamine from the intestinal tract. This absorption, however, is not ordinarily associated with symptomatology.

Somewhat in opposition to the apparently negative physiologic response evoked in experimental animals to the enteral introduction of histamine solutions, Carnot, Koskowski and Libert⁹ found that the subcutaneous injection of from 0.7 to 1.75 mg. histamine stimulates gastric secretion in man, although there was a latent period of from 30 to 60 minutes intervening between the introduction, and the detectable effect of the drug. This action seems not to have been due to nervous control, because Ivy and Javois¹⁰ obtained a similar secretory effect in a "denervated" Heidenhain pouch. It is claimed furthermore by Lim, Matheson and Schlapp¹¹ that if precautions are taken to prevent the admixture of the gastric and duodenal contents, the flow of gastric juice only is stimulated.

There seems to be, therefore, a distinct contrast in the response to histamine stimulation between the exquisite sensitiveness of isolated, surviving intestine and the in vivo intact intestine. Nevertheless, persons that are sensitized to certain foods may and do exhibit signs and symptoms that are usually called "intestinal anaphylaxis." It is not to be construed, however, because of this phenomenon, either that intestinal anaphylaxis is necessarily a localized intestinal phenomenon, or that histamine, or a histamine-like substance, is necessarily a factor in this anaphylactic syndrome.

Attention is directed at this point to the fact that histamine, as it is usually available, is an acid salt. The more common preparations are the dichloride, and the diphosphate. These are really very acid. Thus 30 mg. of a preparation obtained from a well known firm required almost exactly 5 cc. of N/20 NaOH solution to bring its reaction to the neutral point, P_H 7.0. Ergamine phosphate, a very reliable British preparation, contains only about one third by weight of the amine: this must be considered in discussing the potency of histamine preparations. The acid salt may be less reactive than the free base.

⁴ Ibid., 1919, 39, p. 497.

⁵ Kotake and Konishi: *Ztschr. Physiol. Chem.*, 1922, 122, p. 230.

⁶ Oehme: *Arch. f. exper. Path. u. Pharm.*, 1913, 72, p. 76.

⁷ Frey and Kraut: *Ztschr. physiol. Chem.*, 1926, 157, p. 32.

⁸ O'Connor: *München, med. Wchnschr.*, 1911, p. 1439.

⁹ *Compt. rend. Soc. de biol.*, 1922, 86, pp. 575, 670.

¹⁰ *Am. J. Physiol.*, 1926, 71, p. 604.

¹¹ *Edinburgh M. J., N. S.*, 1923, 30, p. 265.

Returning to certain anatomic features of the problem of histamine action on the small intestine, it will be apparent at once that a histamine solution applied to a piece of isolated intestine suspended in a Tyrode bath comes in contact first with the serous, rather than the mucous surface. Conditions are very different when the intestine is in its normal position in the living animal. Here, the usual enteric administration of substances is on the mucosa and only after a substance has passed through the epithelial barrier can it reach the underlying smooth muscle. Furthermore, substances that pass into the mucosa may be carried away rapidly through the blood or lymph vessels. To this extent, therefore, the results of tests on isolated intestinal strips cannot be carried *pari-passu* to the mucosa. It should be remarked in passing, also, that histamine solutions poured on the serous surface of exposed, but intact intestines in the animal do not appear to lead to a noteworthy response.

The undoubted importance of histamine and histamine-like substances produced by microbic activity in the intestinal tract, and, also, possibly in so-called intestinal anaphylaxis, has made it quite interesting to reinvestigate the effects this group of powerfully reactive amines may have upon the small intestine when they are applied, not to the serous surface, but directly to the mucous surface of this organ. It has already been stated that the effects hitherto recorded in *in vivo* experiments have not been striking enough to cause more than negative comment.

The literature is singularly barren of records of experiments of the kind undertaken here. One of the earliest references is to Tyrode's work¹² on the effect of cathartics on intestinal motility. He used a length of intestine, bathed in Tyrode solution, through the lumen of which fluids could be passed. A thread, attached to the middle of the piece of intestine, and to a recording lever, writing on a smoked drum was the recording device. This apparatus, suitable for the purpose it was to serve, was not applicable to the study of histamine effects on the mucosa, nor were the improvements made in it by Heer¹³ and Lasch,¹⁴ sufficiently precise and adjustable for this study.

A series of trial models, however, resulted in the piece of apparatus described below, which, while it is still susceptible to improvement, answers the purpose for which it is intended very well indeed.

¹² *Arch. internat. d. Pharmacody. et de Therap.*, 1910, 20, p. 205.

¹³ *Ibid.*, 1911, 21, p. 321.

¹⁴ *Biochem. Ztschr.*, 1926, 169, p. 292.

Description of Apparatus.—The diagram of this apparatus (fig. 1) shows the details of construction and of operation. The principle involved is to suspend and securely fasten a suitable strip of intestine, some 5 to 6 cm. in length, over one end of a long U-tube in such a manner that access of fluid to the mucosal surface is unimpeded, without creating intrainestinal pressure, and without admixture of the bath solution. This is important. The other, free end of the intestinal strip is closed tightly with a strong ligature. This closed, upper end, finally, is con-

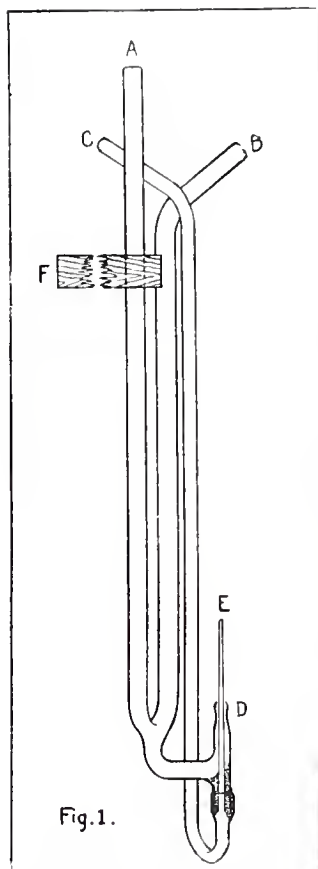


Fig. 1.—Apparatus for applying solutions directly to the mucosa of a strip of isolated, surviving intestine: A, solution inlet; B, air inlet; C, solution outlet; D, inlet into gut; E, outlet from gut; F, holder. About $\frac{1}{2}$ size.

nected in the usual manner with one end of a writing lever, whose arms have a ratio of 1:8. This apparatus is suspended by the holder F. in a Tyrode bath and holding 150 cc. of solution kept at body temperature by an electrical device.

Operation.—One end of the intestinal strip is slipped carefully over the small tube E (which opens through its continuation C to the outside air) and over tube D, far enough to permit of ligation at the construction, shown clearly in the text figure. Neither air nor fluid can enter or escape at this end of the intestinal strip. The other end, tied, and suspended as above indicated, com-

pletely shuts the lumen of the piece of intestine from all contact except through tubes A or B, at one end, and the small tube E, through C, at the other end. Suitable solutions can be introduced to the mucosal surface of the intestine up to the level of tube E without causing more than minimal intraintestinal pressure. In practice, the test fluid is introduced through tube B: after the fluid has entered tube A is closed by a clamp, and sufficient air pressure is applied above the solution in tube B to overcome the capillarity. The fluid passes easily to the desired level in the intestine, and exerts its pharmacodynamic effect, which is recorded on a smoked drum in the usual manner. The amount of solution actually in the lumen of the intestine from the ligation at D to the level of E is very little: probably not more than 0.5 cc. About 3 cc. of solution must be introduced through B to fill the system from the bottom of the U-tube to this point, however.

Care must be taken in passing the strip of intestine over the small tube E. If the mucosa is injured, the effects are indicated by the promptness with which the muscle shortens in response to the drug. Usually, however, the strip slips over very smoothly, if the precaution is taken to hold the piece of gut vertically during the operation.

Fluid that has been introduced may be removed from the lumen of the intestine, not quantitatively, but sufficiently so for ordinary purposes, by simply reversing the procedure of introducing the fluid in the first place. This frequently is an advantage.

It may be stated that strips of small intestine from the guinea-pig thus suspended in warm Tyrode solution retain their contractibility about as long, and as effectively, as those similarly suspended in accordance with the Trendelenberg technic.¹⁵

Normal salt solution, or Tyrode solution (warmed to body temperature), or other physiologically neutral fluid may be introduced and withdrawn from the lumen of the intestine without causing a discernible change in its length or reactivity. The physiologic condition of the intestine may always, of course, be observed by the simple expedient of adding some suitable contraction-inducing substance, such as histamine, to the Tyrode bath.

EFFECTS OF HISTAMINE SOLUTION ON THE INTESTINAL MUCOSA

Nature of the Solutions.—Unless otherwise stated, the histamine solutions used throughout these experiments contained 0.25 mg. per cc. by weight of the acid salt, which had previously been made neutral by the addition of the requisite amount of N/20 NaOH. The exact amount of the free base histamine in such a solution cannot be estimated for reasons mentioned above. One-tenth cc. of this solution added directly to the Tyrode bath always elicited an immediate and decided contraction of the intestinal strip, if it was in good condition. This contraction could be recorded on the smoked surface of the drum in the usual manner. This is a control upon the contractability of the intestine under observation.

¹⁵ Arch. f. exper. Path. u. Pharm., 1917, 81, p. 53.

Controls.—Warm, normal salt solution, and warm Tyrode solution failed to elicit any change in length of the piece of gut, when they were introduced into the lumen. Dilute acid (0.25% lactic) and dilute alkali, (1% sodium bicarbonate) also had no appreciable effect upon the preparation. Usually, but not always, unneutralized histamine solutions of the strength indicated, that is, acid salts of histamine, either failed to induce shortening of the intestinal strip, or at best a very slow shortening, when they were introduced to the mucosal surface. This was particularly the case when the intestinal contents were acid. Such solutions, however, incited the usual abrupt, powerful contraction when they were added directly to the Tyrode bath. It will be recalled that the Tyrode bath is alkaline in reaction, P_H 8.0 to 8.2, and heavily buffered. The acid histamine salt therefore is changed immediately to the free base when it diffuses into the bath fluid.

The acid salt will not unite with aldehyde in faintly acid solutions and it is not unreasonable to expect the NH_2 group of the amine, which seems to be very directly associated with the contraction-inducing effect of histamine, to be ineffective under such conditions.¹⁶

It is worthy of mention that histamine solution will not induce contractions in isolated, surviving strips of intestine if a very dilute formaldehyde solution (0.1 cc. neutral formalin) is added to the Tyrode bath before the amine is introduced into the lumen of the intestine. This is in harmony with experiments on anaphylaxis, reported in the preceding study.¹⁷

It is worthy of mention, although no quantitative evidence is reported here, that the reaction of the contents of the guinea-pigs' intestines varied considerably. Some were found to be quite alkaline to litmus, others quite acid to the same indicator. Also, and this may be significant, younger guinea-pigs, weighing up to 300 to 325 gm. were found to be more reactive than older, larger animals weighing up to 1,050 gm.

The subjoined reproductions of kymograph tracings of actual experiments are more illuminating than any description can possibly be. The intestinal strip is its own indicator, and tells its own story. A brief discussion, however, will elucidate some details that are significant. It should be stated that the time intervals are uniformly three seconds. The tracings are reduced to approximately one-fourth dimensions.

Experimental.—Figure 2 is a kymograph record of a control experiment. Acid histamine solution was introduced into the lumen of an intestinal strip whose contents were distinctly acid to litmus, at A. After a minute, during which no shortening of the muscle occurred, 0.1 cc. of the same solution was added to the Tyrode bath at B. An immediate and characteristic shortening took place. At

¹⁶ Kendall: J. Infect. Dis., 1927, 40, p. 689.

¹⁷ Kendall, Alexander and Holmes: Ibid., 1927, 41, p. 137.

the height of contracture 0.1 cc. neutral formalin solution was added to the Tyrode bath at C. The usual rapid relaxation took place, which is shown very clearly.

This experiment shows that in this particular strip of intestine, unneutralized histamine solution, of which 6 mg. of the acid salt required 1 cc. of N/20 NaOH to bring it to p_H 7.0, applied directly to the mucosa failed to induce a contraction. A small amount, added to the alkaline, buffered Tyrode bath, however, and therefore applied to the serous coat of the intestine as the free base, induced a prompt contracture.

Figure 3 illustrates the effect of the same acid histamine solution on a successive piece of the same intestine, in which the mucosa was intentionally dragged over the end of the small tube E, in figure 1. It was hoped that sufficient injury would thereby be produced to make a portal of entry for the solution through the mucosa to the underlying muscular tissues. It will be seen that almost as soon as the solution was introduced into the lumen, the contraction began. It rapidly increased and became so intense the lever went off the drum. Formalin was added to the Tyrode bath at the height of contracture, as in experiment 2, and after a very brief interval the muscle relaxed. It is worthy of mention that in this experiment the contracture-exciting substance, the histamine, entered the smooth muscle from the mucosal side, and the relaxing substance, the formaldehyde solution (0.1 cc. of neutral formalin) passed from the Tyrode bath into the serous side. It is striking how quickly these opposite effects induced from opposite sides of the intestinal strip take place.

Figure 4 is a reproduction of a kymograph tracing, in which neutralized histamine solution was introduced into the lumen of a strip of intestine at A. In about 40 seconds, the strip shortened; not rapidly but steadily, and with increasing speed. When the contraction was very marked, but not at its maximum, the usual formaldehyde test was applied, by adding 0.1 cc. formalin to the Tyrode bath. The relaxation was very prompt, and complete. Here again, as in experiment 3, the histamine solution was applied from the mucous aspect of the intestine and the formalin from the serous aspect. The effect of the neutral histamine solution thus applied, seems unequivocal. There is a considerable interval, during which the muscle is quiescent; during this period the reagent presumably penetrates the mucosa. Then as more and more histamine passes through, the contraction begins, and increases progressively.

Figure 5. This experiment was performed in the manner of number 4, with the exception that the formaldehyde solution (one part neutral formalin to 100 parts Tyrode solution) was added to the mucosal surface of the intestine after withdrawing the residual histamine, when the contraction was maximal. It will be seen that there was a sudden, partial relaxation followed by contraction; less, however, than the original. A second application of the formaldehyde solution brought about a second relaxation, which also was followed by a small shortening of the muscle. Finally, 0.1 cc. of formalin, added outside to the Tyrode bath relaxed the strip of intestine considerably, but not to its original length. This phenomena has been noticed several times.

It appears that formaldehyde relaxes contractions induced by histamine solution, but that the effects of the aldehyde are rather more pronounced when they are induced from the serous side than from the mucous side of the intestine.

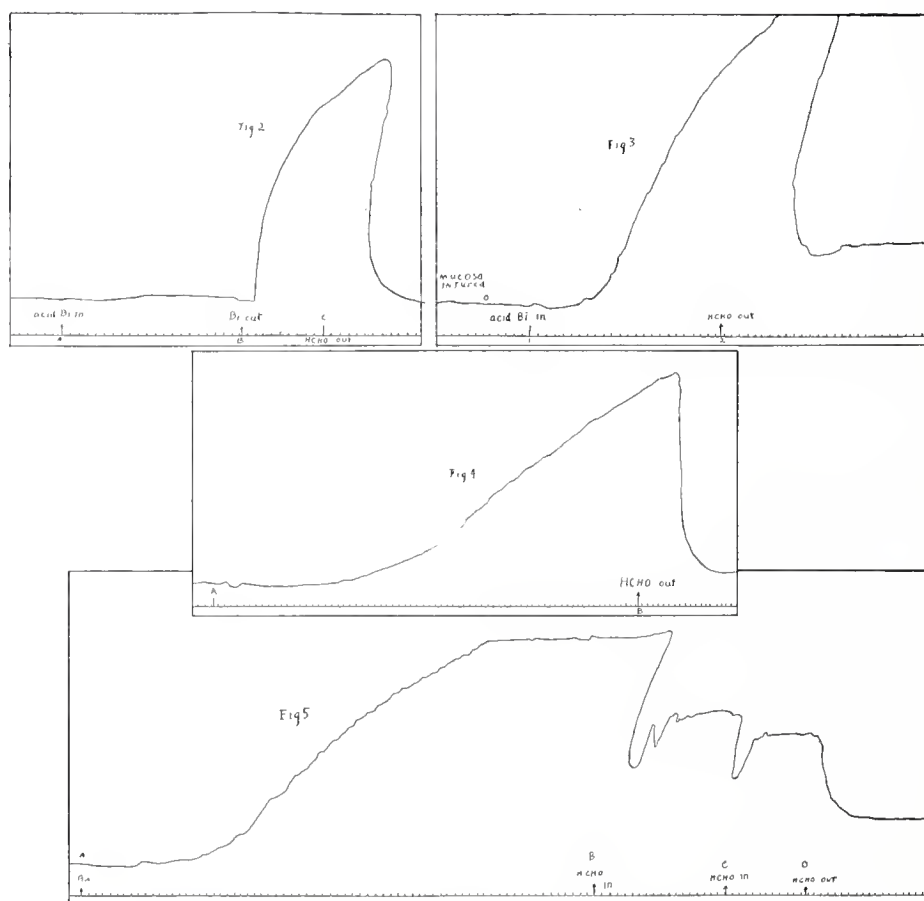


Fig. 2.—Acid histamine (histamine diphosphate) introduced into lumen of intestine at A, no reaction. After 60 seconds, 0.1 cc. of the same solution added to the Tyrode bath at B, immediate and pronounced contraction. 0.1 cc. formalin added to Tyrode bath at C, contraction immediately relaxed.

Fig. 3.—Mucosa intentionally injured. Acid histamine (histamine diphosphate) introduced into lumen of intestine at 1, almost immediate contraction. 0.1 cc. formalin added to Tyrode bath at 2, contraction immediately relaxed.

Fig. 4.—Neutral histamine solution introduced into lumen of intestine at A. After about 30 seconds, contraction begins, and increases progressively. 0.1 cc. formalin added to Tyrode bath at B, contraction immediately relaxed.

Fig. 5.—Neutral histamine solution introduced into lumen of intestine at A, contraction starts in about 30 seconds, and increases progressively. Formaldehyde solution (1 part neutral formalin to 100 parts Tyrode solution) introduced into lumen at B, prompt, partial relaxation, followed by secondary contraction. Formaldehyde added to lumen again at C, relaxation followed by contraction. 0.1 cc. formaldehyde added to Tyrode bath at D, prompt relaxation, but not to original level.

Figure 6 is illustrative of a series of experiments in which acid histamine solutions were introduced into the lumens of intestinal strips in the usual manner, and left for 2 or 3 minutes. The muscular response to the acid salt in these instances was either quite negative, or, at best, by a rather slow, progressive, but slight contraction. If, after exposure for 2 or 3 minutes to the acid histamine, the solutions were withdrawn from the lumen, and 1% bicarbonate of soda was introduced instead, the strip of intestine frequently, but by no means always, responded quite promptly by contracting in a series of progressive waves. Sometimes the progressive contraction was the more marked phenomena, as is shown in figure 6. Sometimes the rhythmic contractions were more conspicuous as is shown in figure 7.

Neutral formaldehyde solution usually relaxed the contraction in the one instance, and stopped the rhythmic contractions in the other. The effect of the aldehyde in relaxing the contraction induced by the histamine, shown in figure 6,

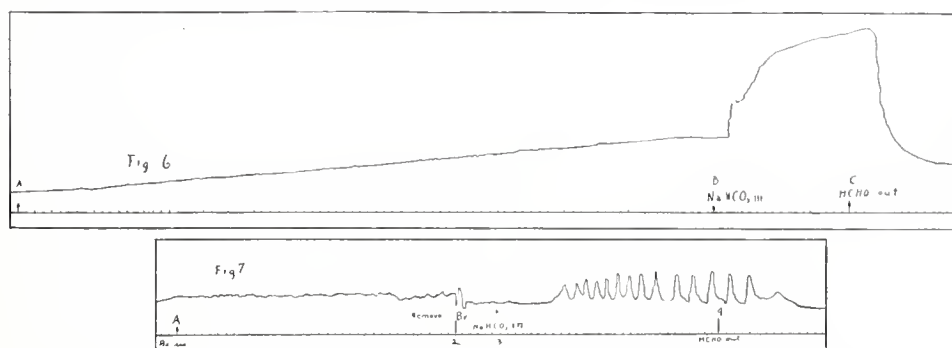


Fig. 6.—Acid histamine solution (histamine diphosphate) introduced into lumen at A, very slow contraction. After 4 minutes, 1% NaHCO_3 solution introduced into lumen at B, prompt contraction. 0.1 cc. formalin added to Tyrode bath at C, prompt relaxation.

Fig. 7.—Acid histamine solution introduced into lumen at A, almost no reaction (irregularities in line due to bubbles of oxygen). Histamine solution removed from lumen at 2, and 1% NaHCO_3 solution introduced at 3. Regular, rhythmic contractions, with but little shortening of muscle strip. 0.1 cc. formalin added to Tyrode bath at 4, rhythmic contractions soon cease, and muscle relaxes.

(Tracings reduced to one-fourth original dimensions)

is reasonably explained by the change of the NH_2 group of the histamine to a $\text{N}:\text{CH}_2$ group.¹⁸ The gradual cessation of the rhythmic contractions, shown in figure 7 when the aldehyde is added to the Tyrode bath, is not so easy of explanation. The phenomenon is being investigated further.

DISCUSSION

The kymograph tracings of experiments on the effects of applying histamine solutions directly to the mucosa of isolated, surviving intestinal strips from guinea-pigs presented herewith are representative of results obtained in a long series of investigations on this subject. The

¹⁸ Kendall: Proc. Soc. Exper. Biol. & Med., 1927, 24, p. 316.

earlier trials, it is proper to state, were irregular, inconclusive, and unsatisfactory. This was due partly to the imperfections of the earlier models of the apparatus, and partly to lack of dexterity in the several manipulations involved. With the newer, and improved apparatus, however, and the acquisition of manipulative dexterity, the results became increasingly uniform. Nevertheless it is freely admitted that they never became mathematical in their precision. On the whole, the later experiments are reasonably conclusive in about three out of four trials. Probably the percentage of positive results will be increased somewhat with improvements in the apparatus, but it is safe to predict perfection will never be attained until some of the idiosyncrasies of smooth muscle are better understood.

Several experimental facts have been elicited from this series of observations, which seem to throw some light on the effects of histamine in the intestine of the guinea-pig. First, it appears that the isolated, surviving small intestines of younger animals are somewhat more responsive to the action of this aromatic amine than those of older animals. Second, the free base is more consistently reactive, when it is applied to the mucosa of the isolated surviving intestine, than the acid salt. Additional evidence upon this point is presented through the effects of adding alkali to acid histamine salts, which, while by no means wholly conclusive, are very suggestive of the fact that the free base is more reactive in inducing contractions than the acid salt.

It cannot be stated with finality at this time whether the free base is more reactive because it penetrates the mucosa more rapidly, or because the acid salt is so stable it dissociates slowly in the tissues, liberating the base at a rate comparable with the ability of the cells to adjust themselves without noteworthy response. In this connection, it will be recalled that intravenous injections of histamine solutions are well tolerated, and without effect on blood pressure if they are introduced very gradually. The fate of the histamine, whatever the rate or path of introduction is without the scope of this study.

Dilute formaldehyde solutions, added either to the Tyrode bath, or to the mucosal surface of isolated, surviving small intestinal strips, relax histamine-induced contractions. If such a dilute solution is added to the Tyrode bath, before histamine solution is applied to the lumen of the gut, contraction usually fails to take place. The same phenomenon is observed if the aldehyde solution is introduced into the lumen of the gut before the histamine solution. This is in agreement with previous experiments.

There is a marked difference in the speed of reaction, both with respect to the appearance, and the rate of contraction, when suitable histamine solutions are applied to the serous surface of the isolated, surviving gut, on the one hand and to the mucous surface of the gut on the other hand. In the first instance, the response is prompt, and the contraction abrupt. In the latter instance, 30 to 60 seconds intervenes provided the mucosal surface is uninjured, and the contraction takes place slowly. This is construed as indicating that the absorption through the mucosa is a relatively slow process, and that the effect is on the underlying smooth muscle.

The question may well be raised at this point; is it not possible or quite probable, that even in the most careful experiments, the mucosa is slightly injured, and that the effects observed are really due to slow leakage of the amine through a wounded surface. The rejoinder is that this is very distinctly a possibility. Attention is directed to three facts, however. The histamine solution introduced induces an abrupt contraction, when the mucosa is injured, as is shown in figure 3. Also, acid histamine solutions usually fail to induce characteristic contractions, when they are applied to the mucosa, but the subsequent addition of alkali seems to provoke an immediate, or almost immediate response. Finally, it should be remembered that as little as 0.1 cc. of the histamine solution employed for mucosal stimulation will elicit a prompt and vigorous contraction, if it is added to the Tyrode bath, as is shown in figure 2.

Nevertheless, this objection is a very valid one, and its significance is fully appreciated. The slowness of the contraction following the application of a suitable histamine solution to the mucosa of these isolated, surviving strips of gut seems to explain plausibly the negative results obtained in *in vivo* experiments. The histamine may well be slowly absorbed in the living mucosa, but it is probably carried away in the blood stream so rapidly in proportion to the absorption there is little chance for it to pass to the underlying smooth muscle in threshold stimulating amounts.

This at once focuses attention on the possible effects of histamine absorption from the human intestine, where there is relative stasis of circulation. Those cases of constipation associated with an overgrowth of gas bacilli,¹⁹ are apparently of this type.²⁰ These will be referred to

¹⁹ Kendall and Schmitt: *J. Infect. Dis.*, 1926, 39, p. 250.

²⁰ Kendall: *J. Am. M. A.*, 1926, 86, p. 737.

later. Suffice it to say that the removal or suppression of the agent that produces these histamine-like substances improves the condition of the patient materially. It is not without clinical significance that the suppression of the gas bacillus in these cases is rather readily brought about by persistent administration of well soured milk, together with a suitable regimen.²⁰

Finally, one additional important fact requires mention. After an intestinal strip has responded to histamine stimulation from the mucosal side, and the contraction thus elicited has been relaxed with formaldehyde it is usually impossible to elicit another characteristic contraction with fresh histamine solution even after prolonged washing of the muscle that has been subjected to the histamine-aldehyde reagents with Tyrode solution. At best a slight transient contraction occurs. It will be recalled that those muscle strips which have had the histamine applied to the serous surface, can be freed from the histamine-aldehyde reagents by washing with fresh Tyrode solution, and then histamine will elicit a marked contraction.

It seems probable that the muscle has been injured by the passage of histamine-aldehyde through it. There is evidence, which will be reported later, that the histamine-aldehyde compound, although devoid of contraction-inciting properties, is still quite poisonous for the smooth muscle.

SUMMARY

Neutral histamine solutions, applied directly to the mucosa of isolated, surviving intestinal strips, usually induce slow but progressive contractions. These contractions usually become apparent after a latent period of from 30 to 60 seconds. Neutral histamine solutions applied to the serous surface of intestinal strips, on the contrary, induce rapid and abrupt contractions. Acid histamine solutions applied to the mucosa of intestinal strips either fail to induce contractions, or incite very slow, and limited contractions.

The addition of weak alkali to acid histamine solutions in contact with the mucosa of isolated intestinal strips tends to accentuate both the rate and height of contractions.

The intestines taken from young guinea-pigs are in general somewhat more responsive to neutral histamine solutions applied to the mucosa, than the intestines of older guinea-pigs.

The experimental evidence presented herewith points to a definite, but slow, absorption of histamine from the lumen of the intestines through the mucosa. Histamine thus absorbed from the intestinal lumen in the living animal is probably removed through the blood stream as fast as it is absorbed under normal conditions.

Suitable aldehyde solutions prevent, or relax histamine contractions induced in isolated, surviving intestinal strips, depending on whether these aldehydes are applied before, or after, the introduction of the amine.

ANAPHYLACTIC CONTRACTION INDUCED THROUGH THE MUCOSA OF THE ISOLATED, SURVIVING INTESTINE OF THE GUINEA-PIG

STUDIES IN BACTERIAL METABOLISM. LXXXVI

ARTHUR ISAAC KENDALL AND PHILIP LEONARD VARNEY

*From the Department of Bacteriology and Public Health, Washington University School of
Medicine, St. Louis, Mo.*

It was shown in the preceding study¹ that neutral histamine solutions, applied directly to the mucosa of isolated, surviving strips of small intestine of the guinea-pig, through the use of a special piece of apparatus, would induce slowly developing contractions in such preparations, where control, inactive substances, as dilute acid and alkali, and normal salt solution would not. It was also shown that contractions thus induced with suitable histamine solutions, could be relaxed promptly by formaldehyde, and, as shown elsewhere, by other aldehydes as well.² Furthermore, such contractions did not occur in intestinal strips that were treated with very dilute formaldehyde solution prior to the application of the amine solution. Finally relaxation of these histamine-induced contractions could be brought about not only by adding the dilute formaldehyde solution to the Tyrode bath, but also by adding it to the lumen of the intestine as well.

It has also been shown that anaphylactic contractions may be induced, relaxed, or prevented from taking place in isolated, surviving intestinal strips of guinea-pig intestine by the proper use of the specific antigen, and of formaldehyde or by certain other aldehydes.³ The latter experiments were made upon the serous coat of the intestine.

It follows very naturally from these observations, that anaphylactic contractions might be induced in isolated, surviving intestinal strips taken from sensitized guinea-pigs, by applying the homologous antigen to the mucosal surface with the apparatus described¹ in place of applying the antigen to the serous surface, as is customarily done. Also, the effects of suitable aldehydes upon these anaphylactically induced contractions could be advantageously studied in this manner.

Received for publication, May 7, 1927.

¹ Kendall and Varney: *J. Infect. Dis.*, 1927, 41, p. 143.

² Kendall: *Proc. Soc. Exper. Biol. & Med.*, 1927, 24, pp. 316, 492; *J. Infect. Dis.*, 1927, 40, p. 689.

³ Kendall; Alexander and Holmes: *J. Infect. Dis.*, 1927, 41, p. 137.

Anaphylaxis of intestinal origin is usually recognized in association with "food idiosyncrasies," but observations of a direct experimental nature do not seem to be numerous. Of course the inducing of contracture in isolated, surviving organs from sensitized animals—uterus and small intestine especially—has long been known, but in such experiments especially those with intestine, the antigen is applied to the serous, rather than the mucous coat. This is not the natural path for the antigen to follow.

However, Hettner and Krig,⁴ have reported that ingested protein may be absorbed from the alimentary canal, and detected in the serum by direct test, and Anderson, Schloss and Myers⁵ state that the blood serum of normal infants may give a specific precipitin test following the ingestion of certain proteins. This test soon disappears as a rule.

Hektoen, Kanai and Dragstedt⁶ found that fresh thyroglobulin (fresh thyroids) fed to dogs, passed in sufficient amounts into the blood from the digestive tract without losing its specific antigenic properties to be detected by specific precipitin tests. Their observations are important also, because they indicate that at least some of this absorbed, antigenic protein is carried away in the blood stream. This observation is in agreement with the probability that histamine, absorbed from the digestive tract, may also be carried away, in part at least, through the blood stream.¹

With all these possibilities in prospect, a series of experiments was performed in which properly diluted specific antigen was added directly to the mucosal surfaces of strips of isolated, surviving small intestine taken from properly sensitized guinea-pigs. The details and results follow.

Procedure.—Sensitization: Guinea-pigs weighing up to 300 gm. were sensitized by intraperitoneal injections of either 1:5 solution of egg albumen, or 1:3 horse serum, in normal salt solution. Two-tenths cc. of the former, or 0.3 cc. of the latter was usually employed. The animals receiving the egg albumen were tested usually on the 14th day after the sensitizing injection. Those receiving horse serum after the 18th day. The animals were killed by a blow upon the head. The small intestine was removed at once, and placed in a cold room in a covered dish lined by filter paper moistened with Tyrode solution. The animals were more satisfactory to work with, when they were deprived of food for 12 hours before they were killed.

⁴ Am. J. Physiol., 1925, 73, p. 539.

⁵ Proc. Soc. Exper. Biol. and Med., 1925, 23, p. 180.

⁶ J. Am. M. A., 1925, 84, p. 114.

Apparatus.—The usual Tyrode bath, and Trendelenberg technic described previously,⁷ was used throughout this work. The bath held 150 cc. and could be emptied and filled very readily without disturbing the remainder of the apparatus. The device described by Kendall and Varney¹ was found to be very convenient for applying the antigens directly to the mucosa of sensitized strips of intestine.

Records of experiments were obtained in the usual manner upon a smoked drum by means of a lever, whose arms were in the ratio of 1 to 8. One arm was attached to the piece of intestine, the other carried the writing point. Shortening of the muscle is indicated in the subjoined reproductions of kymograph tracings by an upward movement of the curves; relaxation by downward movement of the curves. Time intervals are uniformly in three seconds.

Controls.—Sensitization Controls: A strip of small intestine from a suitable guinea-pig was suspended in the Tyrode bath in the usual manner. One or two cc. of the antigen, diluted as above indicated, was added directly to the bath fluid. If the animal was sensitized, the characteristic contraction was observed, usually after a latent period of several seconds. Figures 1 and 2, reproductions of kymograph tracings, are typical control anaphylactic contractions from guinea-pigs, one of which (1) was sensitized to horse serum, the other (2) sensitized to egg albumen, as above described. These two intestinal strips, it will be seen, shortened rapidly after a brief latent period. At the height of contraction, 0.1 cc. of formalin was added to each. The muscles relaxed promptly. This is quite in accord with similar experiments described in previous studies.³ Such intestinal strips are therefore specifically sensitive, and succeeding strips from the same gut are suitable for a study of the effects of the homologous antigen applied directly to the mucous surface in place of the serous surface. Of course the latter aspect is the one exposed to the antigen in the usual Trendelenberg technic.⁷

Specificity Controls: Egg albumen, in the proper concentration was added to the Tyrode bath in which a strip of intestine from a guinea-pig sensitized to horse serum was suspended, and, similarly, horse serum, in the proper concentration was added to a strip taken from a guinea-pig sensitized to egg albumen. The results, as might confidently have been expected, were entirely negative. There appears to be no "cross sensitization" between the egg albumen and the horse serum in the amounts used herein.

Aldehyde Controls: Inasmuch as formaldehyde, and certain other aldehydes prevent anaphylactic contractions from taking place in isolated, surviving, sensitized strips of intestine, small amounts of neutral formalin 0.1 cc. were added to the Tyrode baths in which definitely sensitized intestinal strips were suspended. It was found uniformly that the subsequent addition of the specific antigen to such preparations failed to elicit a contraction. This again is in confirmation of previous observations.³

Experimental.—Figures 3 and 4, are reproductions of kymograph tracings of contractions induced in strips of small intestine from guinea-pigs sensitized with egg albumen. The antigen was applied to the mucosal surface by means of the apparatus described in the previous study of this series.¹ After a latent

⁷ Kendall and Schmitt: J. Infect. Dis., 1926, 39, p. 250.

period of about 30 seconds the contraction began, and rose gradually but progressively to a relatively high level. Formalin added to the Tyrode bath, and consequently applied to the serous surface of these strips, relaxed the contractions. This relaxation was nearly complete in both (fig. 3). It will be noted that the entire course of these experiments, both with respect to latent period following the application of the antigen, the onset and course of the contracture, and the relaxation with formalin, is strikingly reminiscent of the histamine-induced contractures, using the same technic.¹ Here again as in the histamine experiments referred to, the contraction-inducing substance enters the smooth muscle from the mucosal aspect, whereas the contraction-relaxing substance enters from the serous aspect of the intestinal strip.

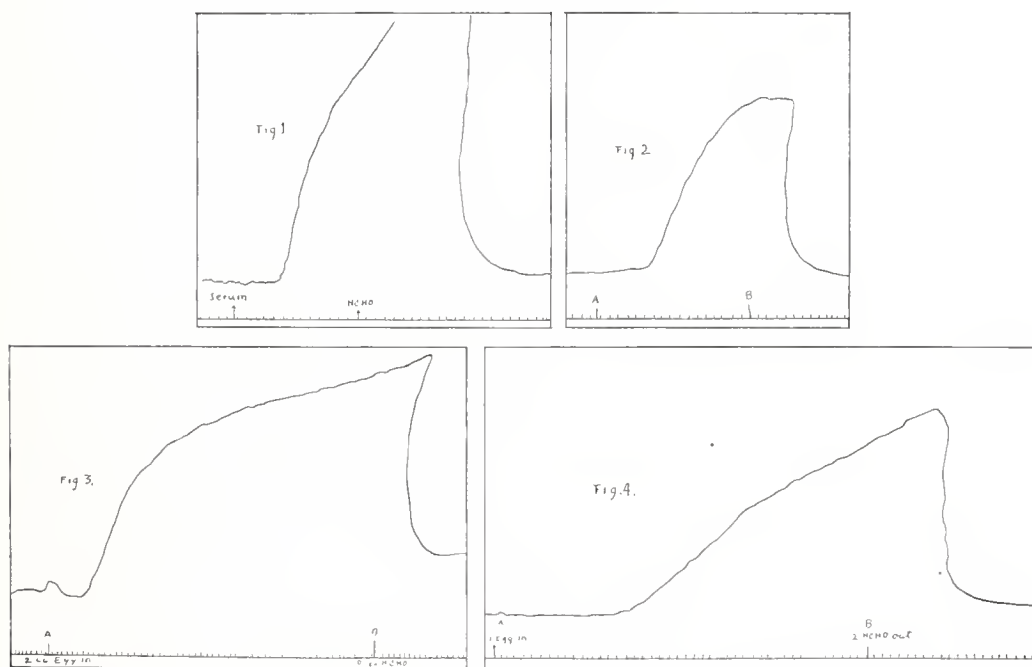


Fig. 1.—Sensitization control. 2 cc. of 1:3 horse serum added to Tyrode bath, contraction typical. 0.1 cc. formalin added to Tyrode bath, prompt relaxation.

Fig. 2.—Sensitization control. 2 cc. of 1:5 egg albumin added to Tyrode bath, contraction typical. 0.1 cc. formalin added to Tyrode bath, relaxation.

Fig. 3.—Egg albumin (1:5) introduced directly into lumen at A. After 30 seconds, a progressive contraction takes place. 0.1 cc. formalin added to Tyrode bath at B, prompt relaxation.

Fig. 4.—Egg albumin (1:5) introduced into lumen at A. After 40 seconds, a progressive contraction takes place. 0.1 cc. formalin added to Tyrode bath at B, prompt relaxation.

Precisely similar experiments were performed, using horse serum and strips of intestine from guinea-pigs sensitized to horse serum, in place of the egg albumen and intestinal strips from animals sensitized to the egg albumen. The results were qualitatively the same, and records of such experiments are therefore omitted.

A few experiments were performed in which the egg albumen was applied to the intestinal mucosa of a guinea-pig sensitized with horse serum and vice

versa. Inasmuch as no discernible reactions took place, and from a priori considerations, no reactions should be expected, the kymograph records of these control experiments are also omitted.

It is worthy of note; indeed, it may be a very significant fact, that in no instance did large guinea-pigs, weighing 500 to 1100 gm. exhibit sensitiveness either to egg albumen, or to horse serum, when the specific antigen was applied to the mucosa of isolated intestinal strips. Such intestinal strips, in several instances, gave fairly satisfactory contractions in response to the application of the antigen to the serous surface of the gut, however. No adequate explanation for this difference in reactivity between young and old animals presents itself at this time. It is quite possible that further attempts might ultimately be successful. The unsuccessful animals numbered four. Three of these were sensitized to egg albumen, one to horse serum.

Previous experiments, with histamine, suggest that histamine dichloride or diphosphate, both strongly acid salts, are less reactive, when applied to the intestinal mucosa of guinea-pig intestine, than the free base itself. The evidence upon this point is rather qualitative than quantitative, however.¹ It will be recalled, also, that the addition of dilute sodium bicarbonate solution to the mucosa that has been exposed to histamine acid salts, frequently gave rise to rhythmic contractions, which are wholly unlike any naturally occurring intestinal waves.

The egg albumen, and the horse serum used in these studies was ordinarily diluted with normal salt solution, which is somewhat acid in reaction— pH 6.8 in several of our samples.

Figure 5 shows an effect elicited in a moderately sensitized intestinal strip, to the mucosa of which horse serum (homologous antigen) was applied at 0. There was no apparent effect in 30 seconds. This was followed by the introduction of a 1 % sodium bicarbonate solution at A. Several marked contractions and relaxations followed, which were succeeded by a rather mild anaphylactic contraction. Histamine, added to the Tyrode bath at B caused the usual abrupt contraction.

Figure 6 shows an effect induced in the succeeding piece of gut, when horse serum dissolved in the bicarbonate solution was added. After a brief period of some 30 seconds, a series of violent contractions and relaxations ensued, followed by the usual anaphylactic contracture, relaxed with dilute formaldehyde solution.

Figures 7 and 8, are kymograph tracings from a similar series of experiments, in which egg albumen was the homologous antigen in place of horse serum. The phenomena are qualitatively parallel, except that the alkaline, Tyrode-egg albumen antigen provoked a persistent rhythmic contracture, rather than a period of rhythm, followed by the anaphylactic type of shortening. Dilute formaldehyde solution as usual, both relaxed the contractions and stopped the rhythm.

Information that is available at present does not suggest an adequate explanation for this rather unexpected effect of weak alkali upon a mucosal surface that is simultaneously absorbing histamine, or an homologous antigen. It is obvious that the degree of alkalinity used here is quite above that amount which ordinarily would be judged normal.

The trend of these experiments, although they are too few in number to permit of generalization, is that the phenomenon of anaphylactic contraction induced from the mucosal aspect of the isolated, sensitized intestine, is apparently furthered when the antigen is applied in an alkaline, rather than an acid medium. It was shown in the previous study¹ that a similar phenomenon was encountered when histamine was applied to the intestinal mucosa, as an acid salt, and as the free base.

One other phenomenon encountered in some of these experiments deserves comment. It was shown previously³ that a strip of intestine

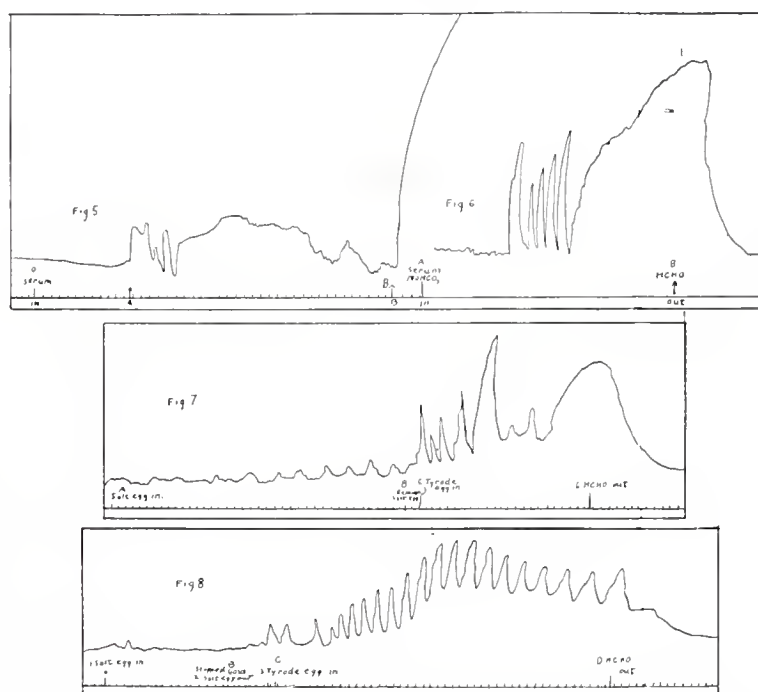


Fig. 5.—Horse serum (1:3, PH 6.8) introduced into lumen at 0, no effect in 30 seconds. 1% NaHCO_3 solution introduced into lumen at A, several irregular contractions and relaxations, followed by mild anaphylactic contraction. Histamine solution added to Tyrode bath at B, prompt characteristic contraction.

Fig. 6.—Horse serum diluted with 1:3 with 1% NaHCO_3 solution introduced into lumen at A. Latent period of about 35 seconds, then a series of violent rhythmic contractions. These are followed by a strong anaphylactic contraction. Contraction relaxed with 0.1 cc. formalin added to Tyrode bath at B.

Fig. 7.—Egg albumin diluted with 5 parts salt solution (PH 6.8) introduced into lumen at A. After a latent period, slow, semirhythmic contractions and relaxations occur. Salt-egg solution removed at B, and egg diluted with 5 parts Tyrode solution introduced into the lumen, quite prompt, violent, rhythmic contractions, with progressive shortening of the muscle. Anaphylactic contraction relaxed with 0.1 cc. formalin added to the Tyrode bath at C.

Fig. 8.—Egg albumin diluted with 5 parts salt solution (PH 6.8) introduced into lumen at A, no effect. After 2 minutes, salt-egg removed, and egg diluted with 5 parts Tyrode solution introduced into lumen, marked, regular rhythmic contractions and relaxations follow almost at once. Muscle gradually shortens. At D 0.1 cc. formalin added to Tyrode bath, prompt cessation of rhythmic contractions, and relaxation of anaphylactic shortening.

(Tracings reduced to one fourth original dimensions.)

from a sensitized guinea-pig could be restrained from contracting in response to the homologous antigen, if a very small amount of neutral formalin were added to the Tyrode bath before the specific protein was added to it. Also, such a muscle was found to be desensitized. If, then, both the aldehyde and the protein were washed free from the muscle strip by several changes of fresh Tyrode solution, histamine would induce a typical contraction in it. It will be seen that the several reagents were applied to the serous aspect of the intestine in these experiments.

In a similar manner, if the aldehyde is applied to the serous surface, and if the homologous protein is applied to the mucous surface of the specifically sensitized intestinal strip, no contraction takes place and the piece of gut is similarly apparently desensitized. Even the most prolonged washing with Tyrode solution however fails to restore the intestinal strip to such a condition it will again respond by contracting to histamine solution.

It seems probable that the passage of the aldehyde from the serous coat, and the entrance of the homologous protein from the mucous coat, injures the muscle to such a degree it is no longer responsive to histamine.

DISCUSSION

The several experiments presented herewith are representative of a very considerable series, in which some 30 sensitized guinea-pigs have been used. This number is exclusive, both of those that were not found sensitized at the proper time, and the larger animals that proved to be refractory. Several successive strips of intestine were available from each guinea-pig, which permitted of the proper controls, as well as the several tests. As was the case with the histamine experiments, some of the earlier experiments were irregular, or negative: when, however, the technical details were more dextrously managed, the percentage of successful experiments was considerably increased.

Several features stand out quite prominently. The most important is that anaphylactic contraction can be induced with a fair degree of precision in strips of small intestine from sensitized young guinea-pigs by applying the homologous antigen to the mucosa. Such anaphylactically induced contractions can be relaxed partly or completely by small amounts of neutral formalin applied either to the mucosa or to the serous side of the contracted muscle.

One of the rather unexpected results of this latter experiment is the rapidity with which the aldehyde relaxes the contraction when the antigen enters from the mucosa, and the aldehyde meets the contraction-

inducing substance, coming in from the serous side. It seems necessary to assume they traverse at least one-half the thickness of the muscular coat to meet in this manner. It might of course be argued that leaks permit the antigen solution to pass to the Tyrode bath, but two facts must be remembered. First, the antigen does not cause an immediate, but a slow contraction. Second, the aldehyde, introduced either from the mucous, or the serous coat, causes an almost instantaneous relaxation. It makes no difference, either, whether the anaphylactic contraction is induced from the serous or the mucous surface, the aldehyde being applied to the mucous aspect: the result is practically always the same.

The several reproductions of kymograph tracings reproduced herewith are qualitatively characteristic of many more, that cannot be published for obvious reasons. They possess one outstanding feature that deserves special mention. This is, their unmistakable similarity to the corresponding histamine curves, presented in the preceding study.¹ This does not mean, of course, that histamine is the long sought for anaphylactic poison. Nevertheless, there are several noteworthy points of resemblance between the anaphylactic and the histamine curves, both with respect to the nature of the response of the muscle in the two sets of experiments and with respect to the action of aldehydes in relaxing the contractions.

It seems not unlikely that the anaphylaxis of intestinal origin, in man, may be due to a process like the one discussed above. Usually, however, one would expect the products of mucosal absorption to be carried away almost as rapidly as they are produced, except in those cases where chronic constipation or other factor slows up the enteric circulation. This, however, lies without the scope of the present contribution.

SUMMARY

Anaphylactic contraction has been induced in isolated, surviving strips of sensitized young guinea-pig intestine by applying the homologous antigen to the mucosa.

These anaphylactic contractions have been relaxed with a dilute formaldehyde solution—when the formalin is added to the mucosa, and—when the formalin is added to the serous surface.

Evidence is presented which suggests that dilute sodium carbonate solution, creating an alkaline medium, aids the anaphylactic reaction.

The reaction induced in sensitized strips of intestine by the homologous protein, applied to the mucosa is suggestively similar to that induced by histamine under parallel conditions.

LEPTOSPIRAS FROM TAP WATER

JOHN E. WALKER

*From the Department of Preventive Medicine and Clinical Pathology, Army Medical School,
Washington, D. C.*

Organisms now classified in the genus *Leptospira* were accurately described and pictured in 1914 by Wolbach and Binger¹ who found them in fresh-water ponds. The later demonstration of morphologically similar organisms as the cause of spirochetel jaundice and possibly also of yellow fever re-awakened interest in the publication of Wolbach and Binger. It is now known that free-living leptospiras occur widely distributed in nature. The cultivation of these water organisms has usually been difficult, principally because mediums used for uncontaminated pathogenic strains were used. Hindle,² however, showed that leptospirae could be cultivated with ease from London tap water. The essential feature of the method was the addition of a small portion of feces to a shallow layer of the tap water, as in a Petri dish.

It is the purpose of this paper to report the confirmation and extension of Hindle's experiments as applied to Washington water, together with some negative experiments relating to the pathogenicity of the organisms.

Cultivation of Water Leptospiras in Mixed Culture.—When 30 cc. of tap water was placed in a Petri dish and a portion of feces about the size of a bean added, leptospiras developed after about 14 days. The dishes were kept at room temperature on a desk, protected from direct bright light. No growth occurred when, in control experiments, autoclaved feces was used. This caused considerable mystification until it was found that the ability of autoclaved feces to support growth could be restored by inoculating it with a loop of *Bacillus coli* from an agar slant. Washington tap water is almost free from *B. coli*. On the average, 1 out of 8 tubes of lactose broth inoculated with 10 cc. quantities of tap water shows gas formation. The manner in which *B. coli* exerts a favorable influence on the growth of the leptospiras was not determined. The dishes not inoculated with *B. coli* usually showed a profuse growth of molds, and were acid in reaction. It seems that *B. coli*

Received for publication, April 21, 1927.

¹ J. M. Research, 1914, 30, p. 23.

² Brit. M. J., 1925, 2, p. 57.

in some manner restrains these deleterious organisms. The possibility however of some metabolic product of the colon bacillus being directly utilized by the leptospiras must also be considered.

It was found that the feces could be replaced by a variety of substances, such as hay infusion, broth, milk, egg, serum, and blood. The concentration of these substances was a very important factor, no growth or poor growth occurring in very concentrated or in very dilute solutions. Egg yolk gave the best growth, with milk (1:20 to 1:100) next. The addition of 0.1% to 0.4% agar aided the growth of the leptospiras, increasing them in number, and shortening the incubation time before their appearance.

A medium of the following composition was the most favorable of several combinations tried: egg yolk, 1 cc.; agar, 1 gm., and tap water, 300 cc.

The agar was dissolved by heat, the egg yolk added and thoroughly shaken after the flask was cool enough to handle, and 20 to 30 cc. of the medium then poured into a Petri dish and inoculated while still fluid with about 5 cc. from another culture. No precautions for sterility were taken. For the initial culture from tap water, 1 cc. of egg yolk was added to 300 cc. of water, and then sufficient melted agar to give a final concentration of agar of 0.3%. The initial cultures were also always inoculated with *B. coli*, though this did not seem so necessary with the egg yolk medium as with autoclaved feces. In this medium leptospiras developed after 4 to 10 days at room temperature, reaching a maximum of from 10 to 100 organisms per microscopic field in about 14 days. Motile organisms remained for about 40 days, that is until the almost complete drying of the medium.

No growth occurred when distilled water was substituted for tap water in the diluted egg yolk medium. Growth occurred, however, when a sodium chloride solution of the proper concentration was used. Before agar was added to the medium, the salt requirement of the organism seemed to be fairly rigid, and in the neighborhood of 0.1% to 0.2% sodium chloride. The addition of agar rendered the organism much more independent of the salt concentration, growth occurring in 0.8% sodium chloride. Calcium chloride in approximately the same range of concentration can be substituted for the sodium chloride.

Attempts to grow the organisms in test tubes or in layers several inches deep in flasks resulted in failure. At best, only a few organisms were found in the upper portion. This failure of growth in deep layers is associated with a difference of reaction as compared to shallow layers. When the egg yolk-agar medium is poured into a Petri dish and inoculated from another culture, there is a slight initial acidity to phenol red during the first few days. After about a week the reaction, however, becomes stabilized at P_H 7.4 to 7.6. The same medium in a

test tube or flask remains acid throughout, except for a very thin layer of alkalinity at the surface. The mechanism of this acid production in deep layers is apparently the same as that of the Russell double sugar medium. This medium contains 0.1% glucose. In the presence of this small amount of fermentable substance, the typhoid bacillus grows with the production of an alkaline reaction on the slanted surface, but produces acid in the depth of the medium. Hall³ and others have shown that the deep acid portion becomes alkaline on exposure to the atmosphere. The same effect can be observed with the egg yolk-agar medium. If some of the deep acid medium is removed and shaken in the air, it immediately becomes alkaline in reaction.

The leptospiras varied considerably in morphology. Two extreme types were observed. The one comprised long slender organisms, the spirals of which were barely visible as a series of bright dots. The other type was shorter and thicker, with the spirals plainly visible. The slender forms usually predominated. Between the two extreme types however there were intermediate forms, apparently forming a gradual transition from one type to the other. This gave the impression that the different types represented morphologic variations of the same organism. It is impossible to be certain of this in mixed cultures of the kind studied. The morphologic variation is not very much greater than that seen in pure cultures of known pathogenic origin. It is my impression that the water organisms cannot be differentiated morphologically from known pathogenic organisms. No attempt has been made to obtain the organisms in pure culture.

On two occasions, efforts to grow the organisms from the Baltimore water supply failed. Cohen⁴ has shown that they are present in Pittsburgh water. The Washington supply is Potomac River water collected at a point about 15 miles northwest of the city. It is purified by sedimentation, coagulation (only when necessary), slow sand filtration, and finally chlorination.

Many other spirochetes of the morphology of the genus *Spironema* (Noguchi) were observed in the mixed cultures. These were particularly numerous in the earlier experiments when the concentration of the egg yolk was greater than 1:300.

Failure to Obtain Evidence of Pathogenicity.—Buchanan⁵ found that leptospiras from the roof of a mine were pathogenic for guinea-

³ J. Infect. Dis., 1926, 38, p. 15.

⁴ From the University of Pittsburgh, personal communication.

⁵ Brit. M. J., 1924, 2, p. 990.

pigs. Zeulzer⁶ cultivated a strain of water leptospiras in serum for a year and found that it had become pathogenic at the end of this time. Spirochetal jaundice, as pointed out by Uhlenhuth and Grossman,⁷ is almost always associated with polluted water or mud. This epidemiologic feature also suggests very strongly that free-living organisms may possibly be related to the disease.

The cultures in the agar-egg yolk medium, containing from 40 to 100 leptospiras per microscopic field, seemed particularly suitable for the demonstration of any possible pathogenicity, even though they were grossly contaminated. However, approximately 50 guinea-pigs, injected with varying quantities of the cultures failed to show any evidence of infection with leptospiras. Intraperitoneal injection of 2 cc. of the cultures resulted in death from peritonitis within 24 to 48 hours. In no instance were leptospiras demonstrated in the tissues of the animals. Surviving animals never showed jaundice. Nor was any evidence of latent infections obtained when animals were killed from 2 to 3 weeks after injection. Ten animals were given repeated small injections of the organisms for several days, likewise with negative results.

It is interesting to note, however, that a case of spirochetal jaundice occurred in Washington while these experiments were being carried on. This case will be made the subject of another report by Towler and Walker.

Cultural Comparison with Pathogenic Strains.—Three strains of pathogenic leptospiras were available, two of *Leptospira icterohaemorrhagiae* and one of *Leptospira icteroides*. Two of these strains were kindly supplied by Doctor Noguchi. One of the strains of *Leptospira icterohaemorrhagiae* was isolated from the patient referred to in the preceding paragraph. When cultures of these organisms were inoculated into the agar-egg yolk medium in Petri dishes and at the same time inoculated with *B. coli*, motile leptospiras were found on microscopic examination for periods of from 10 to 30 days. The organisms were never very numerous, sometimes not more than 1 in 50 fields. No growth was ever obtained when these cultures were transferred to new mediums. This apparent failure of multiplication of the pathogenic strains in the agar-egg yolk medium, and especially the failure of growth or transfers, contrasts sharply with the profuse growth of the water leptospiras.

⁶ Centralbl. f. Bakteriöl., O. 1, 1922, 89, p. 171.

⁷ Klin. Wehnschr., 1926, 5, p. 1113.

SUMMARY

Leptospiras from Washington tap water grow profusely in mixed culture in a medium consisting of egg yolk, 1:300, in 0.3% agar. The presence of *B. coli* in the medium is favorable to the development of the organisms. The necessity for shallow layers of the medium in Petri dishes is apparently related to the fact that the medium in deeper layers acquires an acid reaction.

No evidence of pathogenicity was demonstrated in the organisms so cultivated. Pathogenic leptospiras in the agar-egg yolk medium survive for 10 to 30 days. There is, however, no clear evidence of multiplication, and no growth occurs in successive transfers. This latter feature, under the conditions of these experiments, forms a sharp line of demarcation between the water organisms and pure cultures of strains derived from humans.

POTENCY OF STORED PERTUSSIS VACCINES

LUCY MISHULOW, CAROLYN OLDENBUSCH
AND
MARIE SCHOLL

From the Bureau of Laboratories, Department of Health, New York City

The reports of the value of pertussis vaccine as a prophylactic and curative measure are contradictory. Some¹ are favorable, whereas others² indicate practically negative results. The claim has been advanced that freshly prepared pertussis vaccines are more antigenic than stored vaccines. Thus Huenikens³ reported his results on a series of children whom he immunized with pertussis vaccines. He treated one group with vaccines that were less than one week old; and a second group with vaccines that had been stored for varying periods. Two weeks after the last injection he tested their serums for complement-fixing antibodies. Thus measuring immunity by the stimulation of complement-fixing antibodies, he found that vaccines that had been stored for 2 to 3 months immunized in only about 12.5% of the number tested. On the other hand, freshly prepared vaccines employed within a few days of preparation immunized in about 94%. But the freshly prepared vaccines that he used and those that he designated as stored, were not the same preparations. He used commercial vaccines in his tests of stored vaccines and compared their potency with those that he prepared himself and used soon after preparation. In our experience with bacterial vaccines we find that there may be wide variations in the potency of different lots of vaccine prepared at different times using exactly the same technic. Therefore, in order to compare the potency of fresh and stored vaccines the same preparation should have been employed throughout the test. Others reported favorably on the use of fresh pertussis vaccines as compared with stored vaccines basing their observations on clinical results (Freeman,⁴ Moody⁵ and others).

Received for publication, March 12, 1927.

¹ Graham: *Am. J. Dis. Child.*, 1912, 3, p. 41. Shaw: *N. Y. State J. Med.*, 1918, 18, p. 18. Van der Zande: *Russkaia Klinika (Moskow)*, 1925, 3, p. 147. Bogert: *Am. J. Dis. Child.*, 1918, 15, p. 271. Miller: *Am. J. Pub. Health*, 1921, 11, p. 913. Ladd: *Arch. Pediat.*, 1912, 29, p. 381. Bamberger: *J. Dis. Child.*, 1913, 5, p. 33. Hess: *J. Am. M. A.*, 1914, 63, p. 1007. Hartshorn: *Arch. Pediat.*, 1914, 31, p. 586.

² Barenburg: *Am. J. Dis. Child.*, 1918, 15, p. 273. Von Sholly, Blum and Smith: *J. Am. M. A.*, 1916, 68, p. 1451. Davidson: *Ibid.*, 1921, 76, p. 242.

³ *Am. J. Dis. Child.*, 1917, 14, p. 283; 1918, 16, p. 30.

⁴ *Med. Rec.*, 1920, 98, p. 762.

⁵ *J. Am. M. A.*, 1920, 74, p. 391.

The continuous preparation of fresh vaccines on a large scale is laborious and time consuming. For this reason it is undesirable unless proven to be of greater efficacy than properly stored vaccines. The investigations, therefore, that we carried out have been to determine primarily whether the antibody response of laboratory animals injected with freshly prepared and with stored vaccines would yield any data corroborative of the above claims. The following questions concerning the best method for preparing potent pertussis vaccines were also investigated. Does a freshly isolated strain have greater antigenic value than a strain that had been under artificial cultivation for a number of years? Do vaccines that are grown on chocolate agar differ in antigenic value from vaccines that are grown on Bordet-Gengou medium? Have salt solution preparations the same keeping qualities as glycerol preparations? Does heating the vaccine have any effect on the potency of stored vaccine? Have preservatives any effect on the potency of stored vaccine?

Several lots of vaccines were prepared and tested as follows.

Lot 1.—A strain of pertussis that has been under cultivation for a number of years was grown on coagulated blood agar (chocolate agar) for 48 hours then the growth was divided into two parts.

Vaccine 1: The growth was scraped into sterile 0.85% salt solution, shaken by hand to break up clumps, and heated at 53 C. for one hour. It was then tested for sterility and enough phenol added to make 0.5% the bulk. The concentration was determined by Wright's method and the vaccine was diluted with 0.3% phenol in 0.85% saline to contain 1,000 million bacteria per cc.

Vaccine 2: The growth was scraped into sterile 66 $\frac{2}{3}$ % glycerol in salt solution and shaken in a mechanical shaker for one-half hour to break up clumps. It was standardized by Wright's technic and further diluted with 66 $\frac{2}{3}$ % glycerol to a concentration of 40,000 million bacteria per cc. Phenol was added to make 0.3% of the bulk. Just before using, the vaccine was diluted with sterile physiologic salt solution (which had 0.3% phenol) to contain 1,000 million bacteria per cc.

Lot 2.—Vaccines 3 and 4 were prepared in the same manner as lot 1, with salt solution and glycerol respectively, only a freshly isolated strain was used instead of the old stock strain. The object was to determine, if possible, whether the length of time of artificial cultivation of the strain has any influence on the antigenic value of the vaccine.

Lot 3.—In this preparation a mixture of three old stock strains was used, all of them serologically identical (type A). They were grown on Bordet-Gengou medium for 48 hours and the growth scraped into sterile distilled water. The clumps were broken up by shaking by hand and after the concentration was determined by Wright's method, the vaccine was divided into two parts; one part was heated at 53 C. for one hour. Then an equal volume of sterile 1.7% salt solution was added to both the heated and unheated vaccines thus making the concentration of NaCl in the vaccine 0.85%. Each vaccine was again divided into two parts and phenol was added to alternate lots as follows: vaccine 5, unheated, no phenol added; vaccine 6, unheated, 0.5% phenol; vaccine 7, heated, no phenol added, and vaccine 8, heated, 0.5% phenol.

The object of this vaccine was to determine whether the medium on which the vaccine was grown and the heating of the vaccine in the process of preparation had any influence on the potency of the vaccine. Also, if there is any deterioration during the time of storage, whether phenol has any influence on the rate of deterioration.

Lot 1 (vaccines 1 and 2) and lot 2 (vaccines 3 and 4) were tested for potency as soon as they were found to be sterile, usually within two days, and the bulk was stored in the icebox at 8 C. to 10 C. and tested at intervals for four and one-half years (figs. 1 and 2). Due to pressure of other work at the time, vaccine lot 3 was not tested until after it was stored for 10 months, but after that it was tested at intervals until it was five and one-half years old (fig. 3).

Fresh vaccines were prepared of the strains used in the stored vaccines, and tested at the same time as the stored vaccines in order to control, more or less, the response of each lot of rabbits to immunization (figs. 4 and 5).

Method of Immunization.—A series of 4 to 6 rabbits was used for each of the preparations and each of the rabbits received 250 million bacteria intravenously for the first dose, followed by two more doses of 500 million each at intervals of 4 or 5 days. The rabbits were bled on the seventh day after the last injection and their serums were tested individually for agglutinins, and complement-fixing antibodies. Also the serum of each rabbit was tested before immunization to rule out nonspecific reactions.

Methods of Testing.—Agglutination: After some preliminary tests we found that antigens prepared of the old stock strain and of the freshly isolated strain gave practically the same titer of agglutination with all the serums tested. Antigens of the old strain gave somewhat more uniform results and were therefore selected for testing all the preparations. A 24 to 48-hour growth on chocolate agar was scraped into a solution of formaldehyde (0.1% formalin

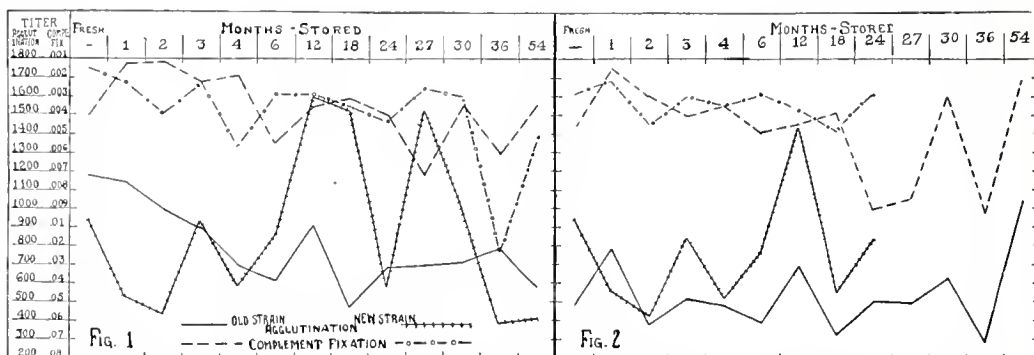


Fig. 1.—Complement-fixing and agglutinating antibody response of rabbits immunized with salt solution vaccines. Growth on chocolate agar; stored and tested at intervals.
 Fig. 2.—Response to immunization with glycerol vaccine, similarly prepared and stored.

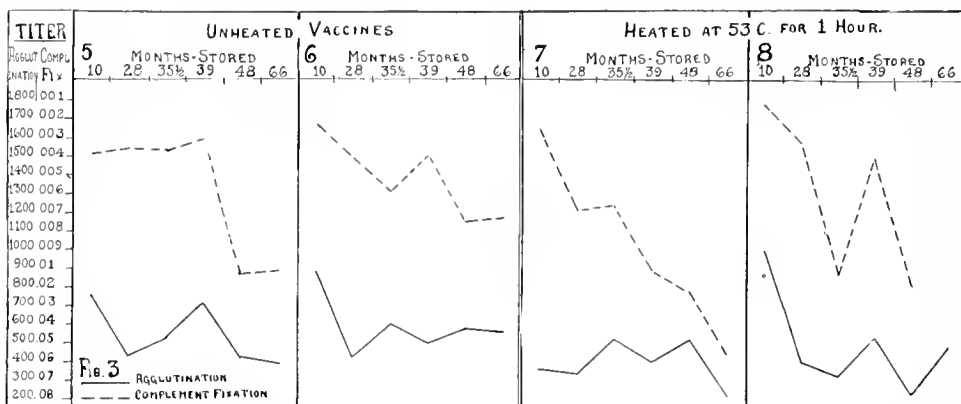
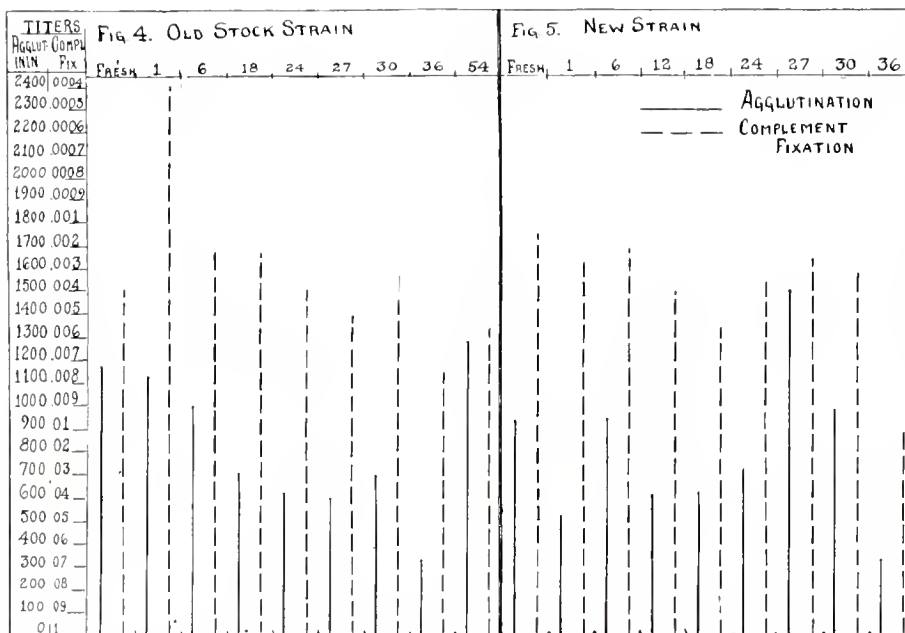


Fig. 3.—Response to immunization with salt solution vaccine. Growth on Bordet-Gengou medium; storage at 8-10 C. Preparations 5 and 6, unheated and heated, respectively, contained no preservative, and 7 and 8 contained 0.3% phenol.



Figs. 4 and 5.—Variations in response of rabbits to immunization with freshly prepared salt solution vaccines. Growth on chocolate agar. These recorded responses were to freshly prepared vaccines, and were used as controls, respectively, for vaccines stored from 1 to 54 months as indicated by the figures of the heading.

in physiologic salt solution) and shaken for a few minutes to break up clumps. It was filtered through cotton and diluted with the formaldehyde-salt solution to the desired concentration, which was determined by preliminary tests. The antigen can be kept in the icebox for a year or more unless it is contaminated.

Serums of normal rabbits frequently gave agglutination in dilutions below 1:50; but in only one instance was there more than a trace of agglutination in a 1:50 dilution. Therefore to rule out nonspecific agglutination, the lowest dilution of the immune serums in our tests was 1:100. The serums were diluted 1:10, 1:20, etc. with physiologic salt solution. Then to 0.1 cc. of each dilution of the serum, 0.9 cc. of the antigen was added and the tests were incubated at 55 C. overnight. The highest dilution of the serum that showed strong agglutination was taken as the titer of that serum.

Complement Fixation: A 24 to 48-hour growth on chocolate agar was scraped into the formaldehyde solution (0.1% formalin in physiologic salt solution). The clumps were broken up by shaking and the suspension filtered through a thin layer of absorbent cotton and centrifuged. The sediment was resuspended in the formaldehyde-salt solution and again centrifuged. The sediment was then suspended in a formaldehyde-salt solution (formalin 0.5%) and shaken for 7 hours in a mechanical shaker. The antigen was titrated with rabbit immune serums and so standardized that four times the amount used in the tests was not anticomplementary and one-fourth of the test dose gave complete fixation with four units of the immune serum.

For the hemolytic amboceptor rabbits were immunized with washed red blood cells of sheep and their serums used as hemolytic amboceptor.

Guinea-pig serum was used for complement. Not all guinea-pig serums could be used in the tests as some gave nonspecific fixation and others gave weak or no fixation with positive serums. It was therefore, necessary to test the serum of each guinea-pig before it could be used in the tests. Four to six guinea-pigs were bled on the morning preceeding the day of the tests. As soon as the blood was clotted and some serum could be obtained by centrifugating, each serum was tested for hemolysis and complement-fixation. Those that were found suitable were pooled and stored until the following morning at a temperature slightly above freezing. The pooled serum was then titrated for the hemolytic unit, and was so diluted that 0.1 cc. contained two hemolytic units.

The serums before testing were inactivated at 56 C. for $\frac{1}{2}$ hour. Each serum of the rabbits immunized with the vaccines was diluted 1:10 and 1:100 with physiologic salt solution and titrations for antibody content were made, using two units of complement and 0.1 cc. of the standard antigen. The tests were incubated in waterbath at 37 C. for one hour to allow fixation to take place and then 0.2 cc. of sensitized sheep red blood cells were added and the tests reincubated for another hour. The hemolytic system was so adjusted that the controls of the serum without the antigen, also of the antigen without the serums, were completely hemolysed in 15 to 20 minutes. The additional incubation of the tests after the controls were hemolysed helped to further rule out weak or doubtful reactions as the unhemolysed blood cells would settle to the bottom of the tubes leaving the supernatant fluid clear in the tubes where fixation was complete. In charting the results, the smallest amount of the serums that gave complete fixation (+++++) was taken as the titer of that serum.

In testing the serums of the rabbits immunized with vaccines lots 1 and 3 for complement-fixing antibodies, an antigen prepared of the old strain was used. The serums of the rabbits immunized with vaccines

of the new strain were tested with both the old strain and the new strain. There was very little difference in the titer of the serums when tested with the two antigens and for the sake of uniformity the results with antigen from the old strain were taken for all the different lots of vaccines.

The serum of each rabbit was tested for complement-fixing antibodies before immunization. In no instance did we get strong fixation in .01 cc. of the serums and only very rarely was there strong fixation in .02 cc. Usually they were either completely negative or gave only partial fixation in .02 cc.

We found considerable variation in the individual response of rabbits in each test. In order to obtain a fair estimate of the results, although each rabbit was tested individually, we used in charting the average response of each series of rabbits in each test (figs. 1, 2 and 3).

DISCUSSION

The results of our investigations which extended over a period of five and one-half years and in which we tested several different lots of pertussis vaccines when freshly prepared and after storing, show that there was no measurable deterioration in their power of stimulating complement-fixing antibodies in rabbits. The vaccine of the old stock strain-vaccine prepared in salt solution (vaccine 1) showed gradual slight deterioration of agglutinogenic power. But it is difficult to draw conclusions from that, as the salt solution vaccine of the newly isolated strain (vaccine 3) showed no such deterioration. The glycerol preparations of both the old stock strain and the newly isolated strain showed no deterioration in their power of stimulating agglutinins and complement-fixing antibodies in rabbits.

There was a marked fluctuation in the response of different lots of rabbits that were immunized with the various preparations as well as individual rabbits in each lot. But these fluctuations were similar in every respect to the fluctuations shown by rabbits when immunized with freshly prepared vaccines tested at different times (figs. 4 and 5). The fluctuation in the agglutinin response of rabbits to immunization with vaccine from the new strain were much greater than in those immunized with vaccine from the old strain; but on the whole they seem to be alike in their antigenic value.

The vaccines prepared of a mixture of three old stock strains which were grown on Bordet-Gengou medium, some of them heated and stored with and without preservative were not tested until after they

were 10 months old. Figure 3 shows that the unheated vaccine without preservative showed no deterioration until it was stored for 48 months. Then there was a decided drop in agglutinogenic power as well as in its power to stimulate complement-fixing antibodies. The same vaccine with phenol gave practically the same results. The heated vaccine without preservative showed a marked deterioration in its power to stimulate complement-fixing antibodies between 10 and 28 months storage, which continued decreasing during the entire period of testing (66 months) but showed practically no decrease in agglutininogenic power. The heated vaccine plus preservative, after an initial drop in both agglutinins and complement-fixing antibodies between 10 and 28 months storage showed no further deterioration, only the fluctuations due to the difference in response to immunization of different lots of rabbits.

The four preparations in this lot of vaccine (lot 3) were tested simultaneously; i. e., the rabbits were immunized simultaneously and their serums were tested on the same day with the same reagents. In this way the differences elicited can not be attributed to possible variations due to the reagents employed. The heated salt solution vaccine plus phenol was found to retain its potency much longer than the heated salt solution vaccine without phenol. The unheated salt solution vaccine with and without phenol retained its potency for a longer period than the heated salt solution vaccine without phenol; but not as long as the heated salt solution vaccine to which phenol was added.

Harvey and Iyengar⁶ in their experiments on pigeons found that agglutinin and complement-fixing antibody response run parallel with the protective power up to 10 months' storage. After that the protective value deteriorates, but the agglutinin and complement-fixing antigenic powers remain high for a much longer period. We could not investigate this claim, as the pertussis bacillus is not sufficiently virulent for laboratory animals to test the protective value of the vaccine. In regard to the production of agglutinin and complement-fixing antibody, however, our results may be summarized as follows.

Pertussis vaccines prepared in salt solution, heated at 53 c. for one hour, containing 0.3% phenol, were found to retain their power of stimulating the production of agglutinins and complement-fixing antibodies in rabbits for a period of from 4½ to 5½ years, when stored at 8 to 10 C. When stored without preservatives such heated, salt solution vaccines deteriorated more rapidly than either the heated vaccine plus preservative or the unheated vaccine. Unheated salt solution vaccine with and without preservatives differ very little

⁶ Ind. J. Med. Res., 1921, 8, p. 715; 1922, 10, p. 192; 1923, 11, p. 110.

from heated vaccines to which preservative was added, when tested after a comparatively short period of storage. On longer storage there was considerable deterioration.

Vaccines prepared of a newly isolated strain of pertussis showed no greater antigenic power than vaccines prepared of an old laboratory stock strain.

Glycerol preparations of both the old stock strain and the newly isolated strain showed no appreciable deterioration in their power of stimulating agglutinins and complement-fixing antibodies in rabbits for a period of $4\frac{1}{2}$ years.

Vaccines grown on chocolate agar did not differ in their antigenic power from those that were grown on Bordet-Gengou medium.

CONCLUSIONS

If the antigenic value of pertussis vaccines can be measured by their power to stimulate specific antibodies, and if the response in human beings to immunization with the vaccines is similar to that in rabbits, properly stored vaccines remain antigenically potent for a considerable length of time. The claim that stored pertussis vaccines lose about 75% of their antigenic potency after storage for 2 to 3 months, has not been substantiated. Eight different preparations, stored at 8 to 10 C. and tested periodically, were highly potent at the end of four and one-half years.

TISSUE HYDRATION AND ITS RELATION TO SUSCEPTIBILITY AND IMMUNITY

AS SHOWN BY SKIN TESTS IN ASTHMA, CHRONIC SINUSITIS
AND OTHER INFECTIONS

WILLIAM B. WHERRY

From the Department of Bacteriology and Hygiene, University of Cincinnati, Cincinnati, Ohio

In any discussion of susceptibility or immunity to infection we have been accustomed to pass over the initial stages of the process and to begin our discussion of what happens in the tissues after the bacteria have arrived there.¹ We should be interested first of all in how bacteria gain entrance to the tissues, for in order to do so they must penetrate into either skin or mucous membrane. Let us focus our attention on the factors which make possible the invasion of this mass of colloids, and largely emulsion colloids, which we call the animal body: We cannot be satisfied with the current mode of approach to this subject in which most writers seem satisfied with saying "the bacteria having gained entrance to the body through some suitable path of entrance make their way into the blood or lymph stream or into the tissue spaces—and if the conditions are favorable—and if the resistance of the host is such or so—they grow and multiply and set up an irritation of the tissues." I have not really quoted the above statement but believe it fairly represents the current mode of evading the plain statement that we have very hazy ideas concerning the first steps in the process of infection.

So far as the microorganism itself is concerned, in order that it may lead a parasitic existence it must be provided with a type of respiration, or acquire it through adaptation² which will enable it to survive in the relatively low tension of oxygen found in the tissues of a living host; and it must be possessed of ferments which will enable it to utilize the sources of carbon and nitrogen furnished by the host. Further, it must find the food substances in solution (as after trauma), or be able to produce chemical changes which will bring about a solution of the tissue, for, under normal conditions the cells of the host are composed of emulsion colloids containing little or no free water. This idea of the colloid state is based on the study of soaps and other colloids by M. H. Fischer³ and

Received for publication, May 11, 1927.

¹ Wherry, W. B.: *Am. J. Ophthalmol.*, 1923, 8, p. 11; *Cincinnati J. Med.*, Dec., 1925.

² *J. Infect. Dis.*, 1921, 28, p. 249.

³ *Soaps and Proteins*, 1921.

is supported by his electric conductivity experiments.⁴ That bacteria cannot utilize foods unless they are in the sol state can be readily shown by growing them in nutrient solutions containing increasing quantities of a colloid which is capable of holding water, like agar agar. With certain concentrations of agar agar the bacteria grow very poorly on the gel but luxuriantly in the water of syneresis (food in solution); they produce rapidly spreading growth within a diluted nutrient gel, but do not multiply, or do so very slowly, within a more concentrated nutrient gel. In quite an analogous manner the bacteria which are capable of producing an extensive local edema are the species which spread most rapidly in infected tissues, e. g. *B. pestis*, *B. anthracis*, *B. tularensis*, *B. welchii*, *B. oedematis maligni*, the streptococci of erysipelas, of scarlet fever, of epidemic sore throat, of strangles in horses, etc.

The mechanism by which these bacteria produce the hydration of the tissues which enables them to grow and multiply is not clear. In a few instances we know that in the filtrates of suitably prepared broth cultures there are substances which produce local congestion and edema, e. g., in those of *B. diphtheriae*, *Strept. scarlatinae*, *Strept. erysipelatus*, *B. welchii*, etc. While such culture filtrates may have an antigenic value it does not follow necessarily that the antigenic and edematogenic substances are identical. The edematogenic substances might be amines. For example in a search for the substance responsible for the urticaria in persons sensitive to contact with ascaris, Ransom, Harrison, and Couch⁵ found a non volatile, relatively thermostabile substance which was present in the albumin fraction and which could be absorbed from the acidified albumin fraction by Lloyd's reagent. However, they were unable to free it from the reagent in an active form.

In order to further elaborate the idea that the edematogenic substance might be an amine it may be further noted that as Koessler⁶ has pointed out in a discussion of the relation of proteinogenous amines to medicine, Berthelot and Bertrand⁷ (1912) isolated *B. mucosus* from the human intestine and showed that it could decarboxylate histidine, tyrosine and tryptophane. In the same year Mellanby and Thwort⁸ found that a member of the typhoid-colon group was able to produce histamine from histidine. Koessler and Hanke,⁹ working with

⁴ Science, 1923, 57, p. 724; Kolloid Ztschr., 1923, 33, p. 131; *ibid.*, 1924, 34, pp. 97, 140; *ibid.*, 1924, 35, pp. 138, 294; Kolloidchem. Beihefte, 1926, 23, p. 200; Kolloid Ztschr., 1926, 40, p. 303.

⁵ J. Agricult. Res., 1924, 28, p. 577.

⁶ Proc. Inst. Med., Chicago, 1920, 3, p. 46.

⁷ Cited by Koessler and Hanke, *ref.* 9.

⁸ Cited by Koessler and Hanks, *ref.* 9.

⁹ J. Biol. Chem., 1919, 39, p. 539.

synthetic mediums, found that 6 out of 29 strains of *B. coli communis* split 50% histamine from histidine in the presence of suitable carbon and nitrogen (e. g., glycerol or glucose, and NH_3 salt). Barger and Dale¹⁰ in 1911 showed that the "vasodilatin" isolated by Popielski from tissues contained histamine and adopted the view of Mellanby and Thwort that it was formed by the action of bacteria.

I may further point out that Allen Eustis¹¹ was the first to demonstrate the probable etiologic rôle of histamine in bronchial asthma and urticaria. Dilute solution of histamine applied to the skin of man produces urticarial wheals in a few minutes. Eustis¹² also showed that when histamine was put in contact with the ground up liver of a turkey buzzard it was detoxicated—for the filtrate of such a mixture was no longer capable of producing anaphylactic shock in the guinea-pig.

We have found that histamine hydrochloride does not produce urticaria when applied to the skin of all animals. Among animals known to suffer from so-called "spontaneous" urticaria one finds the horse, cow, dog and man. Tashero and Wherry¹³ in 1924 tested a series of amines on the dog and obtained marked local skin urticaria, with histamine hydrochloride, pyridine, and dimethylamine; and an urticaria of less degree with beta-naphthylamine and phenylendiamine hydrochloride; they obtained negative results with amido phenol, sulphanilic acid, meta phenelenediamine, alpha nephthylamine, diphenylamine hydrochloride, ortho-toluidine, and hexamethylene tetramine. In other words they produced urticaria with those amines with which proteins are most greatly hydrated as shown by M. H. Fischer.

While histamine produces urticaria in the dog and in man it does not in the cat, rabbit, guinea-pig or mouse, nor did it in a capuchin monkey ill of osteomyelacea. This seems to point to a species selectivity in the action of amines.

It is interesting to note that when a 1:1000 solution of histamine or pure pyridine is applied to a scratch on the skin of a dog, the skin for some distance central to the scratch is so altered that an urticarial wheal may be produced by simply scratching the skin with a needle. This probably corresponds to the dermatographia seen in cases of so-called "spontaneous" urticaria and in angioneurotic edema. In addition to the local urticaria produced by pyridine the heart beat of the dog is greatly accelerated. That urticaria may be primarily of central sym-

¹⁰ Cited by Koessler and Hanke, ref. 9.

¹¹ Am. J. Med. Sc., 1912, 143, p. 862.

¹² Biochem. Bull., 1915, 4, p. 97.

¹³ Unpublished data.

pathetic origin is evident from the work of Rosenow¹⁴ on herpes zoster and from the well known effects of adrenalin and of atropin.

In connection with the work I am about to report it is an important question as to whether the local urticaria or edema produced by the introduction of dead bacteria into the body is due to the simultaneous introduction of preformed amines, or on the other hand, to the introduction of more complex proteins which are then split by the body ferments. For if the swelling is due to amines produced by the bacteria we would not expect to be able to produce any immunity to this type of poisoning; on the other hand if it is due to the action of body ferments on the bacterial proteins then we may hope to be able to produce antibodies which can completely detoxicate the bacterial poisons. Another possibility is that the bacteria produce a more complex substance which is in turn split to the amine stage by the body ferments. Antibody production then would be against the more complex molecule and not against the amine, and might consist in the production of a ferment which is capable of splitting the bacterial product to a nontoxic stage. I have been able to satisfy myself that the repeated inoculation of bacteria to which man is sensitive in the above sense leads to the production of a considerable tolerance to an increased dose; and simultaneously with the establishment of such a tolerance the infectious process is brought under control partly, I believe, through the decreased tendency to hydration of the tissues and partly through phagocytic activity.

Let us look at the effect of hydration of the tissues a little further: The intense edema and congestion which surrounds an infected area is not unlike that produced in local anaphylaxis in the sense that it is composed of hydrated gels. This exudate may favor the infection in several ways:

It provides food in solution for microorganisms.

Many spontaneous localized infections give rise to but little, if any, increase in the opsonin titer of the patient's blood. Is this because the opsoninogens are localized by the surrounding edema? In the same patient small repeated injections of the opsoninogen will bring about a recognizable increase in the opsonin titer.

The exudate dilutes the antibodies, if present, or prevents their entrance into the focus. Opie¹⁵ showed that foreign proteins were held back (localized) in a focus of local anaphylaxis for some time after their injection into a sensitized animal, while they were readily absorbed from the tissues of a normal animal. It is conceivable that conversely such an exudate may interfere with the entrance of antibodies into a focus. The following cases illustrate the point.

¹⁴ J. Infect. Dis., 1916, 18, p. 477.

¹⁵ J. Exper. Med., 1924, 39, p. 659.

Case 1.—Chronic acne of more than a year's duration. Many of the larger abscesses on the face and back contained about a dram of pus. Infection with staphylococcus albus. The patient was given an autogenous vaccine for six weeks without apparent benefit. The injections were given subcutaneously. The same vaccine was then administered daily, as before, but now the antigen in 2 minim doses was injected intradermally. All the smaller lesions healed in three weeks. Now pus from one of the larger lesions was examined and while numerous cocci were present there was very little phagocytosis. A little of this pus was suspended in physiologic salt solution and 1 cc. of this suspension was placed in each of two tubes. To one was added a very small loopful of the patient's blood and then both tubes were incubated for fifteen minutes. Smears showed that in the tube containing blood the leukocytes were filled with semidigested cocci—whereas in the control there was practically no phagocytosis. The patient was then sent to a dermatologist with instructions to empty the abscesses and squeeze blood into them; then they healed promptly. It was this ability to get opsonins into an infected focus which made cupping and Bier's method popular.

Case 2.—Acute infection of both antra. On the second day the profuse white creamy discharge showed enormous numbers of encapsulated pneumococci in pure culture with no phagocytosis. The patient had been given a mixed "cold" vaccine containing 14 species including several strains of pneumococci. The phagocytosis experiment was done as in case 1 using a small loopful of the patient's blood. The addition of the patient's blood led to marked phagocytosis and digestion of the pneumococci. On the fifth and sixth days smears of the pus showed that phagocytosis was taking place and two days later the discharge ceased. Evidently here the immune opsonins prevented extension of the process although at first they were without effect on the bacteria growing on the surface of the mucosa. Whether a slight hemorrhage was responsible for the phagocytosis which occurred later was not noted.

This inability of leukocytes to function as phagocytes in an abscess has been discussed by A. E. Wright.¹⁶ I would offer the interpretation that the local edema with the accompanying liquefaction of tissue in an inflammatory focus interferes with phagocytosis. In order to make this point clear it is necessary to recall that leukocytes maintain their spherical form when suspended in a protein solution like serum or plasma. Only when they have come in contact with a solid surface, or have become adherent to such a surface, (like the bottom of a cover-slip or the surface of an endothelial cell) do leukocytes move by means of pseudopodia. This type of contact motility is also common to many species of amoebae. When phagocytes reach an area where the body gels are hydrated they will cease to move or move very slowly. As Kite and Wherry¹⁷ pointed out phagocytosis takes place at the moment when a leukocyte and an opsonized bacterium come in contact. If the leukocytes cannot move and the opsonins are diluted there will be very little phagocytosis—and what there is will be of the so-called "normal" type which takes place when hydrated leukocytes and bacteria come in contact in the absence of opsonin.

With this tissue hydration theory of infection in mind it seemed to me to be reasonable to suppose that if suitably prepared bacteria or their toxins were injected into the skin one should get local exudation (urticaria) in susceptible individuals and no such response in immune individuals. Also in the case of individuals who have been infected previously by certain species of bacteria, the subsequent injection of such bacteria or their toxins might elicit a still more marked local reaction—like one gets in the Arthus phenomenon, i. e., "local anaphylaxis." The discovery of such a state of sensitivity might explain not only the local edema occurring in asthma but also in those cases of chronic sinusitis accompanied by severe headaches, and in other infections.

The usefulness of the intradermal test has been well established in determining susceptibility to the toxins of diphtheria, scarlet fever and

¹⁶ Studies on Immunization, 1909, p. 458.

¹⁷ J. Infect. Dis., 1915, 16, p. 109.

erysipelas, and in determining previous sensitization to many foreign proteins, and the facts are so well known that it is superfluous to review them here. But the fact that similar methods might be used to determine individual susceptibility or immunity to a given bacterial species has not been emphasized, so far as I know. The usefulness of such a determination is at once apparent to anyone who has worked with the extremely varied flora encountered in infections of the respiratory and intestinal tracts and even in many infections of the deeper tissues. So in order to test the question at issue I have made intradermal tests in a series of cases with antigens prepared from quite a number of species of bacteria which I have encountered in similar cases during the past few years.

Technic.—All the antigens were prepared by removing the bacteria from the surface of the medium with a platinum loop; suspending them in 0.85% NaCl solution; heating at 70 to 75 C for one hour; and preserved by the addition of enough carbolic acid to make 0.5%.

While such antigens have yielded excellent results in the therapy of a large variety of cases in my hands, it is quite possible that other methods of preparation will yield antigens better suited for the intradermal test for susceptibility. As an example I may cite that in the recent study of a case of chronic urticaria persisting for over a year and involving the right eye, right face and neck, I tested the patient with a heat killed staphylococcus aureus which was present in considerable numbers on the chronically congested conjunctivae. Only a slight transient urticaria was produced by 1.5 minims of the vaccine. Then I decided to test the "ectoantigen" of the coccus, prepared according to the method of Ferry and Fischer.¹⁸ This is done by suspending the cocci in 0.85% NaCl and centrifuging until clear. The supernatant fluid, sterilized and preserved by the addition of 0.5% carbolic acid, contains the "ectoantigen." An intradermal injection of 1.5 minims of this "ectoantigen" produced an urticarial wheal which spread to 12 to 15 mm. in diameter and this was followed by marked local congestion and edema. Mackenzie and Hanger¹⁹ found that quite a number of adults were sensitive to extracted (N/100 NaOH) derivatives of both hemolytic and greening streptococci. Huber and Koessler²⁰ were almost inclined to doubt the primary role of bacteria in the etiology of asthma because in many hundred instances they had only 3 or 4 reactions which they could call positive. The bacterial antigens were made according to Wodehouse. I am therefore inclined to continue the use of heat killed antigens injected intradermally.

In order to bring some sort of uniformity into the reading of the results of the intradermal tests I arbitrarily assigned the following values to the signs + or 0:

- ±, slight transient urticaria, subsiding in 10 or 15 minutes.
- +, urticaria at the edges of the injected material sometimes showing decided spread but subsiding within an hour or so and sometimes followed by immediate peripheral congestion which disappears in some hours. This I believe indicates susceptibility, but does not show sensitivity.
- ++, urticarial wheal develops in a few minutes and shows decided spread. An erythematous zone sometimes develops in a few minutes but this may be delayed for an hour or more and is marked next day. This indicates sensitivity.

¹⁸ Brit. J. Exper. Path., 1924, 5, pp. 185, 205; J. Lab. & Clin. Med., 1925, 10, p. 817.

¹⁹ J. Immunol., 1927, 13, p. 41.

²⁰ Arch. Int. Med., 1922, 30, p. 617.

+++, like ++ but the erythema congestion and surrounding oedema are very marked. This indicates very marked sensitivity.

0, the injected material is absorbed without the production of urticaria or spreading erythema. Here of course the site of the injection may be congested for a day or more until phagocytosis is completed.

Rarely, marked congestion would follow when no urticaria had been seen. I have attributed this to placing the antigen too deeply in the skin, and have taken it as a sign of sensitivity. Occasionally the appearance of urticaria may be delayed for several hours.

The accompanying tables show the results obtained in some of the cases tested. It is perhaps unfortunate that all of the cases were not tested with all the antigens. The selection of antigens was largely made on the basis of the bacteriology of the individual case. At times when it was found that cases showing sensitivity, e. g., asthma, reacted with particular violence to certain antigens these were tested on other cases whether they harbored the bacteria at the time or not. Further work along this line may show that certain bacterial infections are most often likely to leave a host sensitized; and inspection of table 1 also points to the commoner groups of bacteria responsible for "colds."

In all the attempts to desensitize or immunize a patient I have customarily adopted the following procedure: an injection of 0.5 minim is given subcutaneously into the back of the upper arm or into the thigh. According to the reaction the dose is either repeated daily or increased by 0.5 minim, avoiding the production of a local reaction that lasts for more than 24 hours. When a dose of 6 or 10 minims is reached this is repeated 2 or 3 times a week. If such a larger dose cannot be attained on account of the continued sensitivity of the patient the smaller doses are given daily.

SKIN TESTS WITH BACTERIAL ANTIGENS

In Asthma and Sinusitis (Table 1).—Patient 1, male, over 50 years old, never had asthmatic symptoms until 2 months ago. Exposure to the fumes of vinegar at his factory brings on a paroxysm at once. Is not skinsensitive to 1:100 glacial acetic but fumes arising from a small piece of cotton wet with the solution and placed on his upper arm brought on a violent attack. The typical jelly like mucus, resembling boiled sago, discharged from the bronchi and sinuses, was full of eosinophiles and many bacteria, e.g. numerous *B. mucosus*, gram-positive staphylococci, gram-negative cocci resembling *catarrhalis* or *flavus*, pneumococci, *B. duplex nonliquefaciens*, influenza-like bacilli, pseudodiphtheria bacilli, and streptococci. The patient gave a marked sensitivity reaction to the autogenous *B. mucosus* but not to the autogenous *B. duplex nonliquefaciens*, pseudodiphtheria and anaerobic streptococci. Following the reactions obtained and shown in the table, the patient was put on a vaccine containing those bacteria which gave a +, ++, or ++++. In the course of a month a dosage of 6 minims was reached and the patient was practically free of symptoms. Exposure to the fumes of vinegar brought on another attack. The vaccine was resumed. The attack did not last long. By the end of the second month the patient was receiving 15 minims of vaccine every other day and was free of

all symptoms. So the patient had acquired a tolerance for a considerable dose of a vaccine one-half minim of which at first gave a marked local reaction.

Patient 2, male, 60 years old, developed asthma 2 years ago while in Florida: had severe attacks, often has to sit up at night, and the sputum was typical, with numerous eosinophiles. In this case tests were made with strains resembling those present in smears of the sputum, and the patient was vaccinated with a mixture of the strains to which he reacted and was practically free of symptoms in 6 weeks.

Patient, 3, male, 25 years old, began to have asthma 11 months ago, 1 month after operation on frontal sinuses and antrums. He never had had asthma or prolonged bronchitis before, but had a severe attack after smelling some fermented peaches. The sputum was typical. Smears and cultures show staphylococci, *M. catarrhalis*, *M. flavus*, pneumococci, streptococci, *B. duplex nonliquefaciens* and *B. aerogenes*. Vaccinated with the strains he reacted to. Patient was relatively free of attacks after 6 weeks but had a recurrence with an attack of acute sinusitis (antrum). Following the establishment of drainage and more vaccine the patient was free of asthma and remained so during three months to date.

Patient 4, male, 7 years old, the son of patient 5 has had several severe attacks in past year, had no asthma in family history and had attack on train few days before examination. Sputum typical with numerous eosinophiles, pneumococci, streptococci, staphylococci, gram-negative cocci, short gram-negative rods, large cocci and diphtheroids. Patient was not sensitive to autogenous *M. flavus*, pneumococci and staphylococci isolated, but was sensitive to other strains as shown in table 1. He was very sensitive to the mixed vaccine, was vaccinated daily for three months, and has had no attacks since beginning the vaccine and during the subsequent two months to date.

I might add that four cases of asthma (3 children 6 to 10 years, and one adult, 48 years old) desensitized with mixed autogenous vaccines made from the aerobic, partial tension and anaerobic flora have remained free of attacks for three years. Two of these cases have had bronchitis twice in this period without asthmatic symptoms or sputum. In each case the treatment was given within a few weeks from the onset of the primary attack and the results seem to point to the advisability of treating these cases at as early a date as possible.

Chronic Sinusitis (Table 1).—Patient 5, male, 45 years old, the father of patient 4, has had repeated attacks of sinusitis, and repeated colds with reinfections of ethmoids or antra, great loss in weight. *B. mucosus* and *M. albus* were isolated from pus from antrum. Considerable improvement followed treatment with vaccines for two months. He reported himself well in three months and in perfect health in five months, weight normal.

Patient 6, female, 25 years old, had had sinusitis and bronchitis since 6 years of age and numerous operations; and was vaccinated with a mixed autogenous vaccine for three months before the tests were made. No history of severe headaches but catches cold easily and always has some nasal and bronchial discharge. Vaccinated for two months with the vaccine based on sensitivity tests. Two colds during this period. Considerable improvement but final outcome still doubtful. Retested 4 months after beginning the vaccine and was found still sensitive to 4 of the 13 species in the vaccine.

Patient 7, female 30 years old, has chronic sinusitis, has been operated on several times for drainage, catches cold frequently and has almost daily severe headaches. After taking vaccine for 6 weeks the patient was so much improved that she stopped taking the injections. During a period of three months in the late winter was free of headaches and colds and then had a reinfection with return of occasional headaches. On the whole is more comfortable than at any time during past ten years.

Patient 8, female, 48 years old, was in splendid health except for recurrent attacks in left antrum. Pus from antrum in 1926 showed numerous minute gram-negative rods which belonged to the alkaligines group and appears to be related to *B. abortus*. 20 cc. of vaccine chosen according to the tests seems to have given protection during past year.

Patient 9, male, 49 years old, has had very severe frequent headaches for past ten years, with many operations on all sinuses except the sphenoid without more than temporary relief. Mucus blown through nose shows numerous eosinophiles. A year before the tests recorded in table 1 were made the patient was vaccinated for a few weeks with a mixed autogenous vaccine of *M. aureus*, *Streptococcus hemolyticus* and *M. catarrhalis*. The patient went to Europe for

TABLE 1

SKIN REACTIONS TO BACTERIAL ANTIGENS IN PATIENTS WITH ASTHMA AND SINUSITIS, OR WITH CHRONIC SINUSITIS

Bacteria in Antigen	Patients with Asthma				Patients with Chronic Sinusitis					
	1	2	3	4	5	6	7	8	9	10
<i>Micrococcus aureus</i>	+	0	++	0	+	0	+	++	+	+
<i>Micrococcus albus</i>			+	0	+	0		0	+	-
<i>Micrococcus flavus</i>	+		++	0	+	+++	++		±	-
			a							
<i>Micrococcus catarrhalis</i>	++	++	++	+++	++	+	+	0	±	++
<i>Micrococcus meningitidis</i>					a					
<i>Streptococcus hemolyticus</i> (K)...				0	0	+	0		0	0
<i>Streptococcus hemolyticus</i> (B)...	++	-			+	+			++	++
<i>Streptococcus hemolyticus</i> (G)...	++							0		
<i>Streptococcus fecalis</i>						±				
<i>Pneumococcus</i> 1.....	+++	+				++	++	+++	++	0
<i>Pneumococcus</i> 2.....				++	+					++
<i>Pneumococcus</i> 3.....				±	0	0		÷	0	
<i>Pneumococcus</i> 4.....			++	++	±	+				++
Anhemolytic streptococcus (B)*.		++	++	0	0	++				0
		a	a		a (4)					
Anhemolytic streptococcus (K)*.			0			++	+		++	0
							a			
<i>B. influenzae</i> (lung).....				++	++	++				
<i>B. influenzae</i> (spinal fluid).....	+	++	0		±	0	+++			+++
<i>B. mucosus</i> (Friedländer's 6).....	++		+++	+++	+++	+++	+++		++	+++
			a							
<i>B. duplex nonliquefaciens</i>	0	++	-	+	0	+	++	++	+	+++
<i>B. abortus</i> (L).....			++	0	++	+++	+++	++	+++	+++
								a		
<i>B. (K)†</i>			0				±			0
							a			

a = autogenous vaccine.

* Nonhemolytic streptococci isolated by Rockwell's anaerobic carbon dioxide method.²¹

† A strictly partial tension nonmotile, polymorphous vacuolated rod; ferments dextrose, saccharose, maltose and mannitol; no acid from lactose; growth pultaceous but not slimy; requires serum or blood. Isolated from four cases of sinusitis. Some cases are very sensitive to it (fig. 1).

three months; had repeated headaches while gone and on his return the same flora was present in material blown through the nose. Very little discharge. Patient was inoculated for two months with vaccine based on the sensitivity tests and has had very few headaches during the past four months, and no nasal discharge.

Patient 10, female, 45 years old, the wife of patient 9, had recurrent colds, and was vaccinated with mixture based on sensitivity tests for two months with apparent protection which has lasted for four months after cessation of treatment.

²¹ J. Infect. Dis., 1924, 35, p. 581.

*In Mucous Colitis, Urticaria and Angioneurotic Edema (Table 2).—*Many cases of mucous colitis, if not all, are at bottom a reaction to sensitivity. Often the jelly-like mucus discharged from the bowels resembles in its consistency and microscopic appearance the sputum in bronchial asthma—differing only in the bacterial flora. Often the eosinophilia is marked (fig. 2).

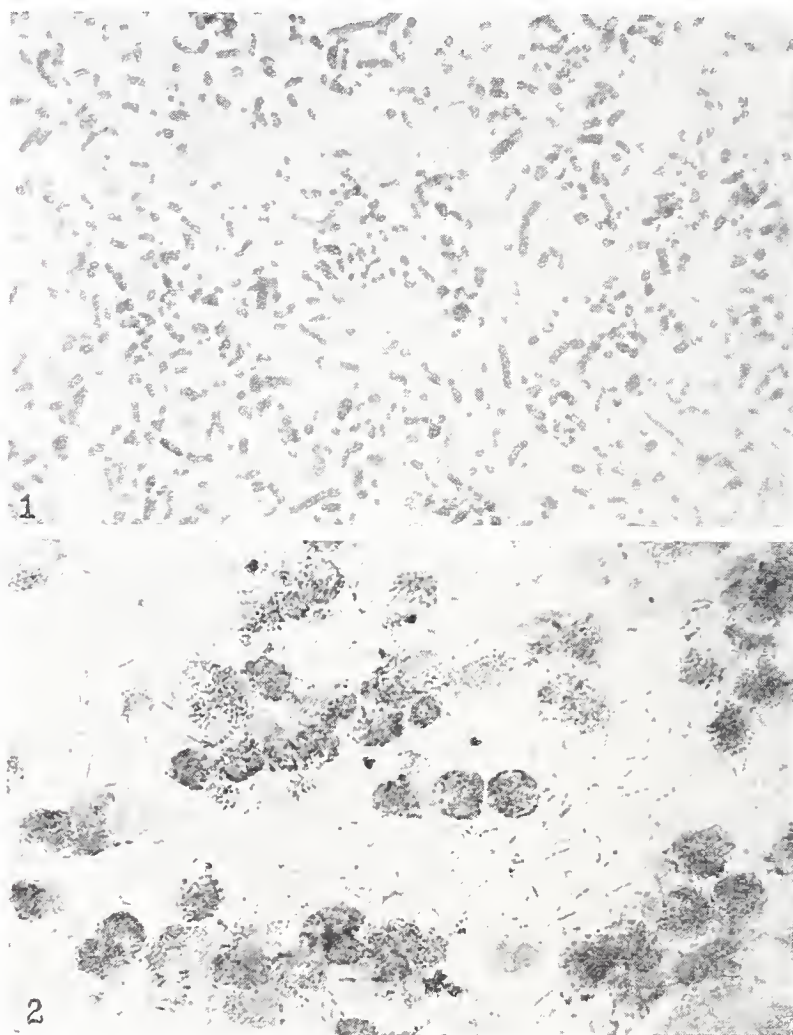


Fig. 1.—Partial tension rod isolated from sinusitis (see footnote to table 1).
Fig. 2.—Eosinophiles in intestinal mucus of mucous colitis.

The etiology of urticaria and of angioneurotic edema is obscure. It is believed that some cases of urticaria at least are associated with what we call food idiosyncrasies. For example it had been noted that urticaria most often followed the ingestion of certain kinds of foods—especially

bloody meats, oysters, fish, etc. These are all foods rich in histidine and if they are hurried down in the undigested state into the colon or if, as shown by Dale and Laidlaw, the upper intestinal tract harbors an abnormal flora containing *B. coli*, histamine production might account for the urticaria, provided the amine passed the liver.

However, as already indicated, it is not necessary that amines should reach the circulation, for if foreign proteins enter in a sensitized individual the body ferments themselves may then act on the protein complex and split off amine—thus giving rise to urticaria. For example: One of our young physicians thoughtlessly gave a case of bronchial asthma a few minims of vaccine intravenously. This was followed within an hour by a most violent attack of generalized urticaria and dyspnea. The patient was not improved by this single inoculation.

TABLE 2

SKIN REACTIONS TO BACTERIAL ANTIGENS IN PERSONS WITH MUCOUS COLITIS, URTICARIA, AND ANGIO-NEUROTIC EDEMA

Bacteria in Antigen	Mucous Colitis				Control "Colds"	Generalized Urticaria		Ocular Urticaria		Angio-neurotic Edema
	11	12	13	14		15	16	17*	18*	19
<i>B. coli communis</i>	±	0	+++	+++	0	0	+++	+++	0	+++
<i>B. coli communior</i>	+++	++	++	+++	0	++	+	+++	+++	+++
<i>B. aerogenes</i>	+++	++	++	±	0	0	+++	+++	±	++
<i>B. acidilactici</i>		+	+	+++			+		±	+++
<i>B. mucosus</i>		+++	++	+++		+++	+++	++	±	++
<i>B. fecalis alkaligenes</i>	+++	0	0	+++	0	+	+		±	
<i>B. proteus</i>		+++	+++				+	+		±
<i>Streptococcus fecalis</i>	±	0	0	+	++	++	+	0	0	±

* Very sensitive to *M. aureus* from eyes.

Since these cases are sensitive to the proteins of one or more of the commoner intestinal bacteria it seems likely that the attacks of urticaria are brought on by the subsequent entrance of the bacterial proteins themselves and that these are then split in the tissues. The fact that desensitization helps these cases would support this idea.

Patient 11, female, 60 years old, with chronic sinusitis and mucous colitis of many years standing, passes large amounts of mucous which contains only a moderate number of eosinophiles, and is very nervous. Stool cultures yielded atypical *B. coli* and *B. aerogenes*. Vaccinated with those bacteria of the respiratory and intestinal series to which the patient was sensitive; put on *B. acidophilus* and lactose by mouth; no improvement in two months.

Patient 12, female, 40 years old, with a mucous diarrhea of long standing, is nervous, has stools of foul odor. Only a few eosinophiles in mucus; *B. coli* and *B. aerogenes* isolated. *B. acidophilus* and lactose by mouth and mixed intestinal vaccine. Only slight improvement in two months and patient stopped treatment.

Patient 13, female, 45 years old has chronic sinusitis and mucous colitis, and is very nervous. After four months of vaccination with the mixed respiratory and intestinal vaccine patient was apparently well.

Patient 14, female, 55 years old, has harbored several *Filaria loa* for many years. Peripheral blood shows 45% eosinophilia. Anemic, nervous, passes much mucus in stools—mucus shows numerous eosinophiles. No intestinal worms or protozoa. *B. coli* and *B. acidilactic* isolated. Put on *B. acidophilus* and lactose by mouth and on mixed intestinal vaccine. Great improvement in two months and blood count (excepting eosinophiles) returned to normal in two months.

Patient 15, female, 56 years old, had almost continuous attacks of urticaria for two years, and intestinal fermentation; is nervous and often passes mucus with stools. *B. coli* and *B. mucosus* isolated. Put on acidophilus and lactose by mouth and given the mixed intestinal vaccine. Considerable improvement of the intestinal condition with disappearance of the urticaria in about two months.

Patient 16, male, 50 years old, has had four attacks of generalized urticaria in past two years; distress after eating, and recurrent sensations of globus hystericus. *B. coli* and *B. aerogenes* in large numbers present in stools. Put on acidophilus and lactose and mixed intestinal vaccine with great improvement. Globus sensation gone and no urticaria during four months observation.

Patient 17, male, 52 years old, has had chronic marginal blepharitis for 16 years; four months ago developed intense urticaria and edema of lids and bulbar conjunctiva of both eyes; marked chemosis and lacrimation, no suppuration. This was complicated by the repeated development of corneal ulcers on right eye which yielded to silver nitrate—but without a reduction of the urticaria. Has periods of partial regression followed by exacerbation. The red velvety conjunctivae harbor large numbers of *M. aureus* and *B. xerosis*. Nostrils also reddened and tender and mucosa harbors very large numbers of *M. aureus*. Skin tests show that patient is extremely sensitive to *M. aureus*. *B. xerosis* produced an urticarial wheal but no congestion. During three periods of spontaneous improvement cultures showed a marked diminution in the number of bacteria present. Edema of eyes relieved temporarily only by wet packs of mg. SO_4 and NaCl .

Patient was so sensitive to the *M. aureus* vaccine that he received only 17 minims during a month by intradermal and subcutaneous routes.

Stool examination now revealed no animal parasites and not much undigested meat. The patient had practically eliminated meat because it disagreed with him. Four cultures made during the next two weeks yielded no growth on ordinary media, i.e., there was practically no *B. coli* in the stools. Patient was put on dilute HCl and acidophilus and lactose by mouth. He was very sensitive to some of the intestinal series and was given a mixed vaccine of these for five weeks. After four weeks of this combined treatment *B. coli communis* appeared in the stools in considerable numbers and at about the end of the third month the urticaria of the eyes disappeared.

Patient 18, female, 47 years old, was in bed from April 10 to August 2, 1925 with extreme urticaria of right eye, right face and neck. When seen on November 6, 1926 still has marked attacks of edema and congestion of right eye and edema of right face, especially at night, and at short intervals. Teeth and sinuses normal. Numerous *M. aureus* on the wet congested conjunctivae, and patient sensitive to this on intradermal test. Tested with intestinal series and found very sensitive to *B. coli communis*. Patient complains of marked gaseous fermentation in bowels. No free HCl in gastric juice. Put on dilute HCl and

acidophilus and lactose by mouth and given mixed vaccine. Great improvement in 4 months to date with long intermissions. Intestinal fermentation still present.

Patient 19, female, aged 23, has had angioneurotic edema since age of 2 and has had at least one attack weekly for as long as she can remember. Familial history of the disease for four generations. Grandfather and an uncle died of edema of glottis. Patient had one attack of edema of glottis while in Cincinnati General Hospital but was brought out with adrenalin without tracheotomy. Attacks of gastric pain with vomiting and swelling of face and extremities—usually once a week at beginning of observations. Patient tested with *M. aureus*, *Streptococcus pyogenes*, *B. coli communis* and *B. coli communior*; very sensitive to *communis* and *communior*. Mixed vaccine given daily. Took a month to desensitize to 0.5 minim. In another month patient could stand 2.5 minims. Increased dosage would produce local areas of superficial extravasation of blood. After vaccination for five months patient had interval of three weeks without an attack. Retested with others of the intestinal series and with the original antigens. No longer reacted to *B. coli communis* but very sensitive to *B. coli communior*, *B. aerogenes*, *B. acidilactici* and *B. mucosus*.

Before an attempt to desensitize further could be made the patient developed mastoiditis and died of septicemia.

SUMMARY

In addition to well recognized qualifications parasites must be capable of freeing water in the tissues. It is suggested that interaction between the parasite and the host leads to the production of amines in susceptible individuals and that the resulting hydration of the tissues favors the growth of the parasite.

Such interaction occurs when heat-killed bacteria are injected intradermally into human beings. The response varies with the species of bacteria and with the individual. The production of local urticaria, or this followed by immediate congestion is taken to indicate susceptibility; when the whole process is greatly aggravated the next day the reaction is taken to indicate sensitivity. Antigens so selected and given by the desensitization method yield excellent therapeutic results due to the production of opsonins and of a lessened tendency to tissue hydration.

A NEW SPIRAL STREAK PLATE METHOD OF ISOLATING BACTERIA BY MEANS OF AN INOCULATING MACHINE

PHILIP L. VARNEY

*From the Department of Bacteriology and Public Health, Washington University School of
Medicine, St. Louis, Mo.*

Surface culture methods of isolating bacteria are generally preferable, not only from the standpoint of simplicity, but also because surface colonies frequently have so distinctive a morphology as to lead to their immediate identification. With the present day methods, however, it is frequently almost impossible to isolate organisms from tenacious masses or from deposits such as tartar. This is often the case from the standpoint of anaerobic bacteriology. Heretofore it has been considered almost impossible to secure pure cultures of anaerobes by other than single cell isolation methods, but that such is not the case has been amply shown by the use of the method to be described.

One of the chief purposes of any isolation method should be to effect a thorough and complete separation of the bacteria in the inoculum, while still maintaining to the greatest possible degree the symbiotic relationship of the various organisms. Failure to achieve this result explains many of the unsuccessful attempts to isolate certain of the more fastidious organisms from badly contaminated material. Several serious obstacles to a thorough separation of bacteria exist in present methods of inoculation, chief of which is the light pressure of application of the needle or rod to the surface of the medium, which is necessary in order to prevent cutting the medium, together with the ziz-zag motion of streaking which is so commonly employed. By such a movement of the needle, clumps of bacteria are removed more frequently than individual organisms, especially in those portions of the plate in which the directions of the motion of the needle or rod is reversed. As a result of this few properly streaked plates are secured, with a consequent increased difficulty in the isolation of the bacteria.

In addition to these objections it is often very difficult to streak all of the surface of the medium due to the contour of the petri dish, with a consequent decrease in the length of the line of inoculation, and in the degree of separation afforded. Furthermore, due to the

unevenness of deposition of bacteria on the plate, none of the present methods permits of an accurate estimate as to the relative numbers of different bacteria present.

In order to overcome these difficulties, a method has been devised in this laboratory, which, tested over a period of three years, has successfully met all tests applied to it, and which in our hands has been far superior for the isolation of both aerobic and anaerobic bacteria than any method previously tried.

The method utilizes a piece of apparatus which has been called the "inoculating machine," a cross sectional drawing of which, with the front battery partially cut away, is shown in figure 1. By means of a suitable interior mechanism, a petri dish which has been placed on the platform A is rotated at a varying speed of 200-400 r.p.m., while the inoculating needle is heavily pressed against the outer edge of the rapidly revolving medium, and then gradually drawn in toward the center of the plate. In this manner a spiral streak is produced, which is many times longer than the line of inoculation on a hand streaked plate, and which is similar in outline to the groove on a phonograph record. The greater number of bacteria are deposited at the edge of the plate, so that the resulting heavy growth occurs in an area which is unsuitable for a proper microscopic examination of individual colonies. A constantly decreasing number of bacteria are deposited along the line of inoculation until finally, near the center of the plate, the most suitable area for a microscopical study of the resulting colonies, the bacteria are deposited only at long intervals. The results of such plate inoculations are shown in figures 4-6. Practically 100% of the surface of the medium is utilized by this procedure, and a far superior distribution of bacteria is obtained than by other methods. As a result of the strong pressure which one may with safety exert against the surface of the medium, and the resulting increased friction, a very efficient grinding of the bacterial mass is obtained, with a consequent increased efficiency of distribution of individual bacteria and a greater degree of ease in securing pure cultures. So great is the pressure one may safely exert against the surface of the medium without cutting the agar that a clean, smooth 22 gauge nichrome wire spud can be bent double. The distribution of organisms is sufficiently even that differential counts of duplicate or triplicate plates usually check within about 10%.

Description of the Inoculating Machine.—Figure 1 is a cross sectional drawing showing the arrangement of the mechanism of the inoculating machine. The model

shown is best suited for construction in the average laboratory, although bevel or worm gear driven models are better and more efficient. Belt driven models have not been satisfactory.

The apparatus consists of a strongly constructed wooden box for enclosing and protecting the mechanism, through the top of which extends a platform (A) on which is placed the petri dish to be inoculated. This platform is driven by a small 2-4 volt battery motor (C*) through the friction drive gears (B and I). The necessary power for operating the motor is obtained from two No. 6 dry

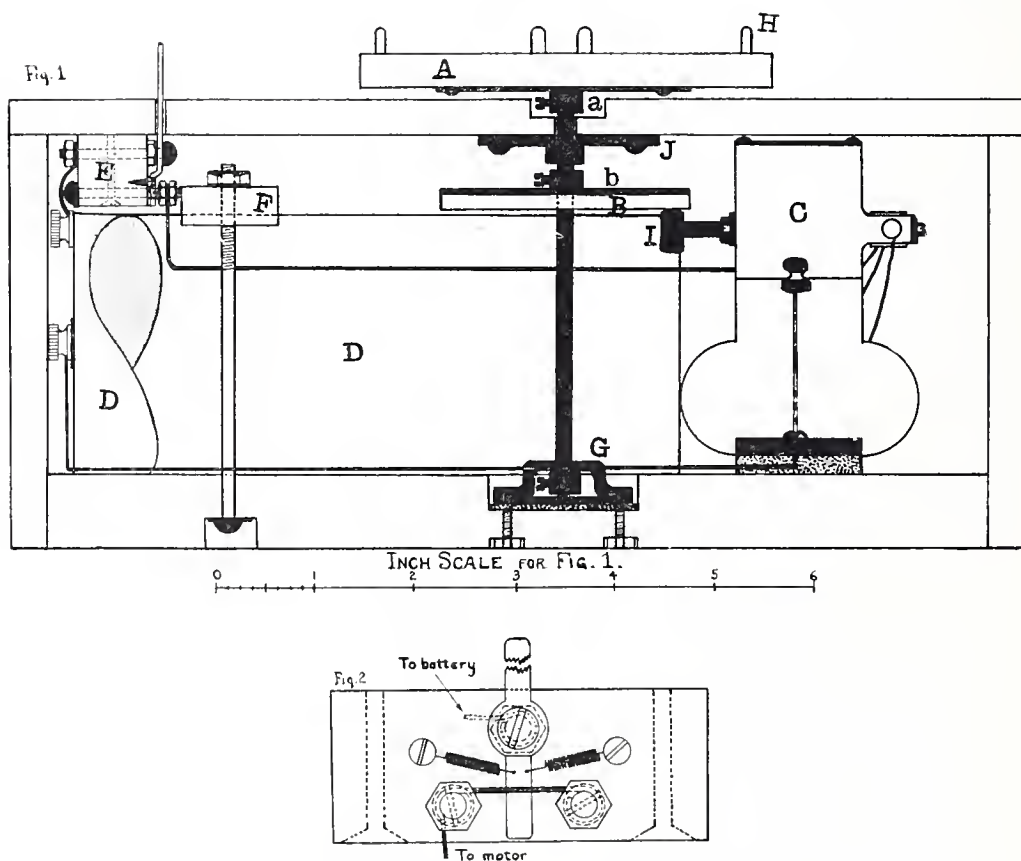


Fig. 1.—Drawing of cross section of the inoculating machine.

Fig. 2.—Front view of switch (E), showing method of construction.

cell batteries (D), which with ordinary use should last several years. The box is so constructed that the end can be readily removed for renewal of the batteries. If portability is not a desirable feature, it is better to substitute a small 110 volt motor** in place of the battery motor, and operate this by means of a foot switch rather than the hand switch used on the portable apparatus.

The rotation of the platform is controlled by means of a switch (E). The batteries are held securely in place by means of a wooden strip (F) which is

* Porter No. 1.

** Knapp Electric Co., No. 710.

bolted to the bottom of the box. Four small lugs (H), set into the outer edge of the platform, prevent the petri dish from sliding off when the apparatus is in motion.

The circular platform is 10.5 cm. in diameter, and may be made from transite or wood veneer. The four lugs, made by cutting off one end of ordinary paper clips, are set into the platform 4.75 cm. from its center, and extend upward 0.7 cm. They are easily placed in position by first drilling small holes in the proper location, filling these with molten sealing wax, and then pushing the heated lugs down into the holes.

Toy Meccano parts may be used in constructing the friction gear, platform and shaft. The platform is made by screwing a steel face plate (a)* to a circular piece of transite or wood veneer, which is then lacquered so as to permit of subsequent moistening of the surface without danger of warping. The $\frac{3}{16}$ inch steel shaft is held rigidly in place by means of two brass bearings, (J and G),



Fig. 3.—Technic of inoculating plates by the spiral streak method.

both made of heavy brass properly drilled and fastened. The latter bearing should be attached to a cork or rubber base to deaden the sound. A small brass collar is fitted to the shaft beneath the gear (G) to prevent all vertical movement of the platform.

The friction gear (B) is made by cementing a steel face plate (b)* to a disc of transite or some other rough faced material. The diameter of this gear is governed by the speed of the motor and the diameter of the gear (I). The platform should revolve under no load at 350-400 r.p.m. The full weight of the platform and gear (B) should rest on the driving gear (I), which is attached directly to the motor shaft. The latter gear may be made by cementing a hollow rubber cork about the pulley wheel, starting the motor, and trimming the wheel to the proper shape with a knife or sharp file. The motor should be so adjusted as to turn the platform in a counterclockwise direction for right handed persons, and in a clockwise direction for left handed persons. As sold, battery motors are connected so that they will revolve the platform in a counterclockwise direction. Reversing the field windings reverses the direction of rotation of the armature.

* Meccano face plate No. 109.

The switch (E) may be either of the toggle switch type, or better, one constructed as shown in figures 1 and 2, and which is made of a wooden block, drilled as indicated, and fitted with brass bolts for the contact members. Such a switch is easily used both by left and right handed persons, regardless of the direction of rotation of the platform, and is especially to be recommended if a reversing attachment to the motor is used.

The base of the wooden case is made of $\frac{3}{4}$ inch thick soft wood, and the top and sides of three ply veneer. This effectually protects and deadens the sound of the interior mechanism. The box should be lacquered or acid proofed so that it can be sterilized with liquid disinfectants without the disinfectant penetrating the wood.

Method of Inoculation.—The method used in inoculating plates is shown in figure 3, which also indicates the method used for controlling the speed of the platform, which consists of pressing one of the fingers against the edge of the rotating platform with varying degrees of tension.

The plate is placed on the platform so that it is securely held by the lugs, the cover carefully lifted, and the motor started. The platform should be allowed to rotate but slowly at first. The inoculating needle is then pressed down against the medium at its extreme outer edge, and held while the plate rotates a few times. The speed is then increased, and the needle drawn slowly inward for a distance of about 1 to 2 cm., or until nearly all of the inoculum has been removed. With the plate revolving at full speed, the needle is then drawn inward more rapidly, so that a wider space between the successive lines of inoculation results than in the first portion of the streak. A constantly narrowing line results, along which the bacteria are deposited at longer and longer intervals, until finally the organisms are separated as indicated in figure 4, which is sufficiently far enough apart to permit of easy isolation.

Further dilution of the material may be secured by first sterilizing the needle, then rubbing it over the outer edge of the first inoculated plate, and inoculating a second plate with this material in the same manner as the first. A typical "second plate" produced in this manner is shown in figure 5. For inexperienced workers, or when using an inoculum containing large numbers of cocci or spreaders, the second plate should always be used.

Proper results cannot be obtained if old, rough or soft wire needles are used, hence only clean, smooth nichrome wire needles should be used. Platinum is entirely too soft to be used to advantage, as it bends under but a fraction of the pressure used in inoculating plates by means of this method. The inoculum should be spread over the surface of the plate by means of a nichrome spud when well isolated colonies are desired, but when a heavy growth of organisms is desired, as in the preparation of bacterial antigens, a nichrome loop should be used. When touched to the revolving medium the bacteria migrate in the direction of rotation of the plate, but as they are caught by the loop, they are held and very gradually and evenly spread over the surface of the medium, assuring a heavy inoculation. A plate streaked by this method and shown in figure 6, illustrates another use to which this method can be put. When using the spud, care should be taken

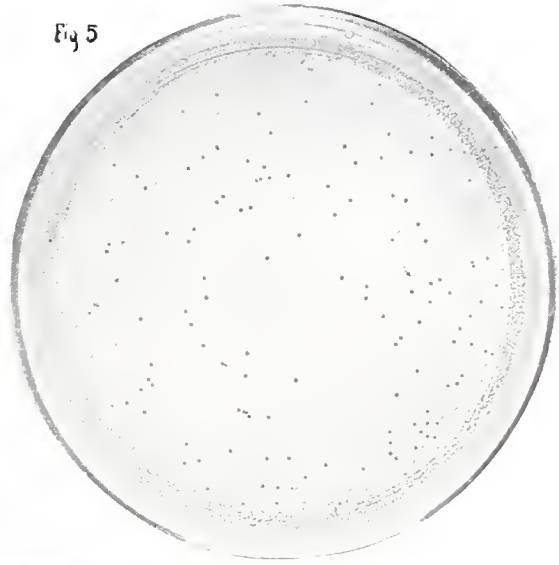
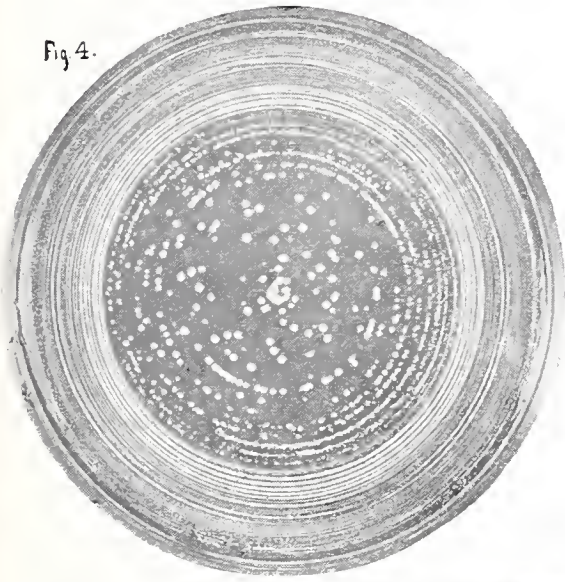


Fig. 4.—A typical isolation plate culture produced by the spiral streak method.

Fig. 5.—A typical second plate culture of *Bact. melaninogenicum* on blood agar showing well isolated colonies.

Fig. 6.—Heavy growth produced by the spiral streak method-plate inoculated with the loop.

to hold it flat against the surface of the medium or the sharp edges may cut the agar. The needle should always be held at a tangent to the surface of the revolving medium. Agar-agar medium, 1.7%, should be used in preparing plates to be streaked by this method, as the centrifugal force developed may disrupt ordinary 1.5% agar-agar or force out drops of moisture during the streaking process.

While designed and originally used for the surface isolation of fusiform bacilli, the spiral streak method is applicable to the isolation of all bacteria, with a far less amount of work than entailed by any other method now in use. It is especially useful in the isolation of anaerobes, and of those organisms customarily considered to be hard to isolate. Spreaders are easily separated from desirable bacteria by its aid.

SUMMARY

To overcome the difficulties and disadvantages inherent in present methods, a spiral streak method of isolating bacteria has been devised, which is performed by the aid of a new piece of apparatus called the "inoculating machine," a description of which is given. By the aid of this method petri dishes are rotated at a speed of 200-400 r.p.m., while the inoculating needle is slowly drawn inward from the outer edge, hence a much longer line of inoculation is possible than by any of the hand methods now in use. A much greater pressure may be applied to the surface of the medium than by hand methods, and as a result of the greater friction so produced, a much more efficient breaking up or grinding of the inoculum is obtained than by hand streaking. The method may be used either for isolating bacteria, in which case a nichrome spud is used, or for producing heavy growths of bacteria for the preparation of antigens, in which case a nichrome loop is used. The method is very rapid, simple and easy in operation.

IS DIABETES OF INFECTIOUS ORIGIN?

EDVARD GUNDERSEN

From the Army Bacteriological Laboratory, Oslo, Norway

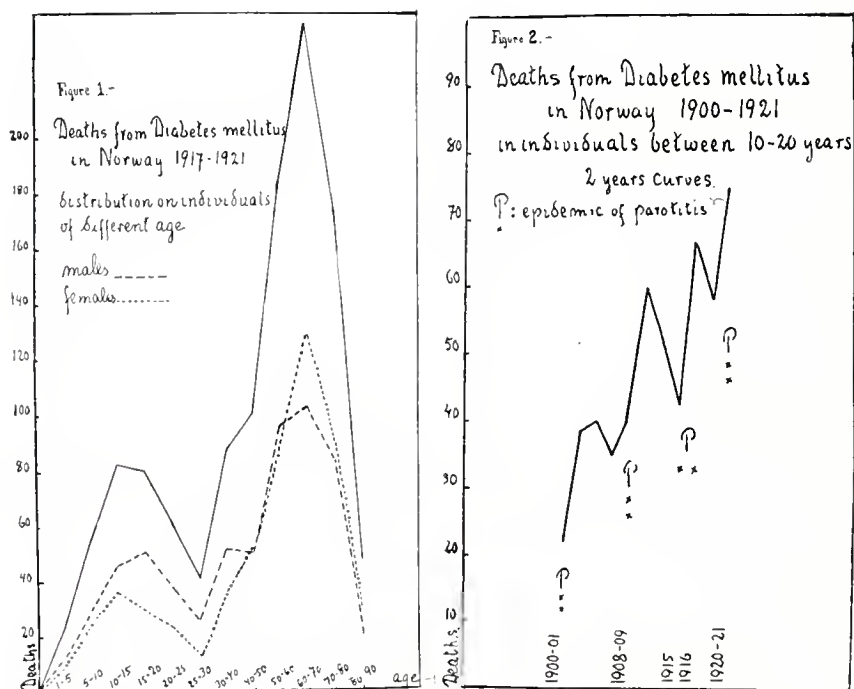
Among the large series of etiological factors ascribed to diabetes mellitus, serious attention has not been paid to its possible infectious origin, inasmuch as diabetes does not bear the stamp of an infectious disease. Yet in late years the epidemiology of diabetes mellitus has frequently been considered, especially since the incidence of this disease has rapidly increased in certain localities, to the extent that its infectious nature has been seriously considered. It is with this aspect of the disease that the following remarks are made.

Figure 1 illustrates the death curve from diabetes mellitus in Norway during a period of five years from 1917 to 1921, the distribution of deaths covering all ages ranging from birth to 80 years of age. Statistical records of deaths from diabetes furnish the most reliable data on this subject, since the deaths recorded give a correct representation of the actual extent of the disease. Of particular interest to us is the distribution of deaths in various age groups, rather than the actual percentage of mortality in each age group. One observes that the deaths from diabetes do not form a continuous curve, but rather two distinct curves dissecting each other. The one represents early life and culminates at puberty, the other more advanced years leading up to the sixty to seventy years' group. It is observed that this peculiarity has occurred every year since 1898, the total deaths recorded in this period numbering 5,951. For convenience, only the last five years' period is considered in this paper, the number of deaths during this period being fairly constant. It is found that in Norway as well as in Denmark, France and America, the increase in deaths from diabetes far exceeds the number expected as a result of the growth in population.

A matter of interest in connection with this curve is that it represents the last period of five years preceding the introduction of insulin as a therapeutic agent. It is possible that insulin treatment in diabetes may modify the typical mortality curve, with elimination of the marked differentiation between the curves of the young and the old patients, and that in the future it may be necessary to reckon with an artificial derangement of the diabetes mortality curve owing to the treatment

with insulin. It is an easy matter of conjecture what this change may mean to anyone desiring to make etiological observations in diabetes on the basis of the mortality curve.

On closer examination it is seen that the mortality curves for men and women follow similar lines both in the younger and older groups. From this observation the inference may be drawn that various influences which at each age may tend to cause diabetes, have like effect on both sexes; that the causes which produce diabetes in the young are not identical with those that produce the disease in the older groups; the



former causes being associable with the individual's age of development, and the latter with the phase of involution of human life. What are these causes? At least some of them are known.

If one studies diabetes in the older groups first, then the actual influence of age must be considered an important etiological factor. In the older groups one encounters the relationship of arteriosclerosis and apoplexy and the causes which contribute to the development of these disease entities have their counterparts in the development of diabetes mellitus, such as heredity, intoxication, obesity, nervousness and miscellaneous disturbances of internal organs and secretions. Pregnancy and the menopause do not seem to cause an increase in the number of deaths

from diabetes among women. Between the ages of 25 and 50 years, when the incidence of diabetes arising from pregnancy should become manifest, one finds that the greater number of deaths occur among men. When the conditional infectiousness of diabetes has occasionally been spoken of it has not been in connection with these older groups. Cases of married people or members of the same household suffering from diabetes within a comparatively short space of time are precluded from consideration, since the probability is that mere chance rather than close infection gave rise to the belief that diabetes is a contagious disease.

In order to avoid confusion in speaking of diabetes and infection, it is necessary to ascertain in what groups infections are encountered. It is well known that infectious diseases, such as scarlatina, typhoid fever and other diseases, may develop diabetes. In cases where diabetes occurs as a complication resulting from an infectious disease, the infection must be classed among the exogenous causes of diabetes. If, on the other hand, diabetes is an infectious disease, one naturally surmises the existence of a specific virus which causes diabetes. What evidence is advanced for supposing that such a virus exists?

The other section of the diabetes mortality curve, namely, the younger group, embraces cases essentially different from those of the older groups. Here we meet with malignant diabetes and occasionally with the form which Strümpel calls "acute diabetes," the duration of which may vary from a few weeks to one or two years. In these instances other etiological factors must be reckoned with than those observed in the milder forms of diabetes. The fact that the curve terminates at about the age of puberty may indicate that the development of sex plays a direct part in the evolution of diabetes in this period. This factor probably only plays an indirect part in the production of the disease. However, it may be seen that the curve rises sharply before puberty and that decisive factors commence their action before sexual maturity. These factors are not definitely known, but perhaps it is possible to discern in these age groups some of the influences of infection which may contribute in the evolution of diabetes mellitus.

In many instances diabetes arises as a secondary condition resultant from pancreatic disorders. The primary condition is met with in pancreatitis. May one suppose the existence of a virus with selective affinity for the pancreas, which causes pancreatitis especially among individuals in the young age groups? One inclines to give answer to this question in the affirmative for sundry reasons. It is well known that in parotitic infection (mumps) the virus attacks certain glands, with

special predilection for the parotids and among others the pancreas, causing acute pancreatitis. Acute pancreatitis following closely on epidemic parotitis (parotitic pancreatitis) has received but slight consideration in the past and has not as yet been seriously discussed as an argument in favor of the infectiousness of diabetes mellitus. This complication is often described in epidemics of parotitis.

As a result of numerous clinical observations, Cheinisse¹ is of the opinion that mild pancreatitis is one of the most frequent complications in parotitis in spite of the absence of histological changes. In 1922 Farnam² made a comprehensive review of literature on this subject and collected 120 cases of parotitic pancreatitis. This condition which primarily may appear as pancreatitis sine parotitide is liable to attack in particular boys and young men.

Hognestad³ has reported three cases of parotitic pancreatitis. In one of these a temporary glycosuria was observed. In another case florid phthisis developed together with polydipsia and subsequent coma, the case terminating fatally. Glycosuria in cases of parotitic pancreatitis is not so rare a disease entity as was previously supposed and is often encountered when it is carefully looked after. As a rule the glycosuria is of a transitory nature, although it is known to have developed into diabetes. The frequency of this condition is not definitely known, since most physicians fail to make a diagnosis of parotitic pancreatitis, the usual complications noted being epigastric pain and abdominal discomfort, cerebral mumps with symptoms of meningitis, associated with vomiting and cholic. The diagnosis becomes more formidable when the case is one of parotitic pancreatitis sine parotitide. This disease entity often brings about secondary damage to the pancreas, especially the islands of Langerhans, and before diabetes manifests itself a more or less long period has elapsed, and the epidemic parotitis is long since forgotten when the young patient is being treated for diabetes. In this state of affairs it has been difficult to assemble a large series of authentic cases of parotitic diabetes. In 1924 Adam Patrick⁴ collected from the literature five cases of acute diabetes following attacks of epidemic parotitis. Clinical indications of pancreatitis was observed in only three of these cases. It has been observed that polydipsia may arise directly from pancreatitis or diabetes, the polyuria making itself progressively manifest about the third or the fourth week of the disease. In 1925 Stevens⁵ reported four cases of mumps of the pancreas in boys six to twelve years old. The onset was sudden, with a rise in temperature, epigastric pain and vomiting on the fifth or sixth day of mumps. Mild icterus was present in two cases. The first description of a case of acute diabetes following on parotitis was given by Harris⁶ in 1899. As early as in 1864, J. Stang made mention in the medical report from the Province of Buskerud in Norway, of a case of diabetes mellitus appearing during convalescence after an attack of parotitis.

The question arises whether it is possible to show any causal relationship between reported cases of parotitic pancreatitis and the appearance of diabetes mellitus in the corresponding age groups? If parotitic

¹ *Semaine méd.*, 1912, 32, p. 85.

² *Am. J. M. Sc.*, 1922, 163, p. 859.

³ *Medicinsk Rev.*, 1925, No. 1, p. 419.

⁴ *Brit. M. J.*, 1924, 2, p. 802.

⁵ *Boston M. & S. J.*, 1899, 140, p. 465.

⁶ *Arch. Pediat.*, 1925, 42, p. 333.

infection plays a rôle in the etiology of diabetes mellitus, one would expect that the extensive epidemics of parotitis, which as a rule occur during a single winter and in particular among boys at the age of puberty, should be followed in the immediately successive years with an increase in deaths from diabetes in the age groups which had suffered the most from the epidemic parotitis. Thus some of the cases of parotitic pancreatitis which occurred during the epidemics of parotitis should reappear in the form of an increase in deaths from diabetes among individuals between the ages of 10 and 20 years, giving allowance of 2 to 4 years from the time of the cessation of the parotitic epidemic. That such is the case is demonstrated in figure 2, which represents deaths from diabetes mellitus among individuals between 10 and 20 years of age during the years of 1900 to 1921. The parotitic epidemics are marked; since they usually commence in October or November and terminate during May or June the following year, the curves have been plotted as 2-year curves. It is observed that the epidemic in 1900-1901 was followed during the successive four years by a marked increase in number of deaths from diabetes, a drop in the curve not taking place before 1906-1907. The epidemic of 1908-1909 was likewise followed by an increase in mortality, a drop in the curve first taking place in 1913. After the epidemic of 1915-1916 the curve rose again and sank in 1919, and following the last epidemic in 1920-1921 it was found to rise again.

From this one observes that the parotitic epidemics have been followed by a rise in the number of deaths from grave diabetes, the form of the disease which terminates in death in the course of about three years. The primary infection of the pancreas which presumptively gave rise to the diabetes must therefore have occurred from three to four years previous to the death of the patient. In this manner the infection coincides in point of time with the parotitic epidemics. And if one should wish to trace the infection farther back, it is only natural to seek for its origin in the parotitic pancreatitis.

The influence that parotitic infection exerts on the development of diabetes mellitus in the young age groups, which for the first time is presented in this paper, does not necessarily exclude the possibility of the rôles other etiological factors may play in the development of diabetes. Eventually, it would seem that the fluctuations in the mortality curve must be considered in connection with the parotitic infection.

Acute pancreatitis is not altogether a rare complication of epidemic parotitis, notwithstanding the fact that not every case of parotitic pancreatitis is accompanied by glycosuria or is followed by diabetes. The

primary alterations caused by inflammatory attacks of the pancreas may heal without traces of secondary alterations in the pancreas. But in the same manner that parotitic orchitis may lead to the atrophy of the testicles, primary parotitic pancreatitis is likewise liable to produce secondary atrophy of the pancreas. Such atrophy of the pancreas is one of the most important histopathological findings encountered in diabetes mellitus, the atrophy being of a granular type of the islands of Langerhans, or a general atrophy of the parenchyma of the pancreas, which may further develop into cirrhosis of the entire organ.

In this manner rapid destruction of the pancreas would seem to be the cause of the graver and more hurried development of diabetes in the young and the slower destruction of the pancreas to be the cause of the milder character of diabetes in the older groups.

SUMMARY

Deaths from diabetes mellitus in Norway during 1898-1921 when distributed in various age groups, form two distinct intersecting curves, one representing early life and culminating at puberty, the other representing more advanced ages leading up to the sixty to seventy years' group, the total number of deaths recorded being 5,951. Mortality curves for men and women follow parallel lines in the younger and older age groups.

Etiological factors influencing diabetes in the older age group are race, heredity, obesity, arteriosclerosis, nervousness and disturbances of internal organs; in the younger age group infections such as scarlatina, typhoid fever and epidemic parotitis, with subsequent parotitic pancreatitis, often lead to grave and rapidly fulminating diabetes.

Parotitic epidemics in the young age group are followed by a rise in the death rate from grave diabetes in the young age group three to four years following each successive parotitic epidemic. Atrophy of testicles following parotitic orchitis is similar in nature to atrophy of pancreas following parotitic pancreatitis.

"Acute diabetes" in youth, graver in type and more rapidly fatal than slower and milder development of diabetes in the older age group, is suggested by the author to be infectious in origin and probably caused by the virus producing epidemic parotitis.

ANAEROBIC MICROORGANISMS IN NASOPHARYNGEAL WASHINGS

INFLUENZA STUDIES. XXXI *

SARA ELIZABETH BRANHAM

From the Department of Hygiene and Bacteriology of the University of Chicago

During recent years several investigators have reported finding anaerobic microorganisms in the respiratory tract.

Conspicuous among these bacteria are the minute gram-negative filter-passing organisms described by Olitsky and Gates.¹ *Bacterium pneumosintes* has aroused special interest because it has been reported as being found only in cases of influenza during the first 36 hours after the onset of the disease. Olitsky and Gates first isolated this micro-organism during the epidemic of influenza which occurred in the winter of 1920, and since that time they and their co-workers have found it in the recurrences of 1922, 1923 and 1926. Partial confirmation of this work has been reported by several investigators,² who have found microorganisms in materials from cases of influenza which were morphologically similar to *Bact. pneumosintes*. A positive agglutination test with *Bacterium pneumosintes* antiserum was obtained in the case of the strain isolated by Hall.³ Olitsky and Gates have described other filter-passing bacteria, both from normal persons and from those with respiratory infections, which they reported as falling into groups 1, 2 and 3. Holman and Krock⁴ and Halland Howitt⁵ have found a small gram-negative diplococcus abundant in the mouths of normal people. This has been generally referred to as *Staphylococcus parvulus* on account of its resemblance to that organism described by Veillon and Zuber,⁶ but Hall and Howitt consider it to be the coccus reported by Leucowicz⁶ in 1901, and in consequence of this have called in *Micrococcus gazogenes*. Tunncliffe,⁷ found *Bacillus rhinitis* in a number of cases of colds, and a peculiar spirochete in a large series of sinus infections. Morphologically the resemblance between *Bacillus rhinitis* and the group 1 of Olitsky and Gates is striking.

During the winters of 1925 and 1926 a study of the occurrence of filter-passing anaerobic bacteria in nasopharyngeal washings from a group of people in Chicago was undertaken. The technic of Olitsky

Received for publication, May 4, 1927.

* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York.

¹ J. Exper. Med., 1921, 33, p. 713; 1922, 36, p. 501. Olitsky and McCartney: J. Am. M. A., 1923, 81, p. 744. Gates and Owens: J. Exper. Med., 1926, 44, p. 787.

² Loewe and Zeman: J. Am. M. A., 1920, 76, p. 986. Gordon: J. Roy. Army Med. Corps, 1922, 39, p. 1. Lister: S. African M. Rec., 1922, 20, p. 434. Detweiler and Hodge: J. Exper. Med., 1924, 39, p. 43. Thomson: Ann. Pickett-Thompson Res. Lab., 1925, 1, p. 253. Hall: J. Exper. Med., 1926, 44, p. 539.

³ Proc. Soc. Exper. Biol. & Med., 1923, 20, p. 280; Am. J. Hyg., 1923, 3, p. 487.

⁴ J. Infect. Dis., 1925, 37, p. 112.

⁵ Arch. de med. exper., 1898, 10, p. 517.

⁶ Ibid., 1901, 13, p. 633.

⁷ J. Infect. Dis., 1913, 13, pp. 280, 289.

and Gates was followed as closely as possible. The nasopharynx was washed out with warm dextrose Ringer's solution, the washings shaken thoroughly with glass beads and passed through Berkefeld V filters with a negative pressure of 40 to 50 cm. Liberal inoculations with 2 to 3 cc. of these filtrates were made in tubes of ascitic fluid containing sterile rabbit kidney and on 5% rabbit blood dextrose agar plates. A vaseline seal was used with the ascitic-kidney medium, and the blood agar plates were placed in an anaerobic jar of the modification devised by J. Howard Brown.⁸ All cultures were incubated for seven days. Several microorganisms were obtained directly from the blood agar plates. Best initial growth was, however, in the ascitic kidney medium. Clouding was evident in many of these tubes, but it was often difficult to see the minute microorganisms in stained smears made from them, as ascitic fluid and autolyzed rabbit kidney present a confusing microscopic picture. Consequently, all of these cultures, after 7 days of incubation, were plated out on rabbit blood dextrose agar, and the colonies so obtained were studied further.

Nasopharyngeal washings were collected from 26 persons, including men, women, and children. There were 8 normal persons, 13 persons with colds, and five with typical uncomplicated influenza. The colds studied were of various types: some were in the early stages; others were of long duration. The cases of influenza occurred during the second week of March, 1926, and were characterized by a sudden onset, aching in back and limbs, fever of 101 to 103 F. and a definite leukopenia, with the leukocyte count about 4,000 in each case. The material from these persons with influenza was collected in every instance within the first 36 hours after the onset. Seventeen strains of anaerobic microorganisms were isolated from 11 of these samples of nasopharyngeal washings. These represented 2 normal persons, 6 persons with colds, and three with influenza; roughly 50% of all persons with colds and influenza, and 25% of the normal persons. Three persons yielded more than one anaerobe; one patient with influenza had four. Of these 17 strains of anaerobic bacteria, 13 seemed, morphologically and culturally, to belong to species previously described. Table 1 lists these organisms; table 2 presents information about the individuals from which they were isolated, and shows their distribution.

Four strains resembled the organisms described by Holman and Krock³ and by Hall and Howitt⁴: 2 were isolated from persons with colds, 1 from a normal person, and 1 from a patient with influenza.

⁸ J. Exper. Med., 1921, 33, p. 677.

They were very small gram-negative cocci, occurring in pairs and clumps, and producing a great deal of gas in the ascitic-kidney medium. They differed from the strains reported by Hall and Howitt in that, like those of Holman and Krock, this gas contained some hydrogen sulphide. On blood agar the colonies were visible with the unaided eye, and were opalescent, glistening, raised, round, and entire. Two strains were spirochetes: one came from a severe cold; one from influenza.

TABLE 1
ANAEROBIC BACTERIA ISOLATED FROM NASOPHARYNGEAL WASHINGS

Microorganism	Normal	Colds	Influenza	Total Strains
{Staphylococcus parvulus or Micrococcus gazogenes	1	2	1	4
Spirochete (Tunncliffe ?)	0	1	1	2
Group 1	0	2	0	2
Group 3	1	0	0	1
{Bacterium pneumosintes or Group 2	1	0	3	4
Minute gram-positive organism	0	1	1	2
Hemolytic diplococcus	0	2	0	2
	3	8	6	17

TABLE 2
THE DISTRIBUTION OF 17 STRAINS OF ANAEROBIC MICROORGANISMS IN VARIOUS TYPES OF
RESPIRATORY INFECTIONS

Condition	Age, Years	Sex	Microorganisms Found
Normal.....	28	Woman	Bacterium pneumosintes, or group 2
Normal.....	23	Man	Staphylococcus parvulus or Micrococcus gazogenes
Influenza.....	45	Man	Bacterium pneumosintes, or group 2; spirochete; Staphylococcus parvulus or Micrococcus gazogenes; minute gram-positive organism
Influenza.....	6	Girl	Bacterium pneumosintes or group 2
Influenza.....	25	Woman	Bacterium pneumosintes or group 2
Old cold.....	30	Man	Staphylococcus parvulus or Micrococcus gazogenes; group 3; hemolytic diplococcus
Old cold.....	50	Man	Group 1; minute gram-positive organism
Old cold.....	40	Woman	Group 1
Acute early cold.....	40	Man	Staphylococcus parvulus or Micrococcus gazogenes
Acute early cold.....	25	Woman	Hemolytic diplococcus
Old cold.....	40	Woman	Spirochete

These spirochetes were strikingly large, both in length and width; they stained irregularly, and seemed full of granules. They closely resembled the spirochete described by Tunncliffe in material obtained from sinus infections. The colonies on blood agar were barely visible with a hand lens, and were quite transparent.

Seven strains were apparently the filtrable microorganisms reported by Olitsky and Gates. Two of these, both from severe colds of long standing, seemed identical with their group 1. The plainly visible

colonies were pointed, and the slender, gram-negative organisms were definitely curved. One strain, also from an old cold, seemed identical with group 3. The colonies were knobbed, and the tiny gram-negative rods were so uniformly granular that they seemed bipolar. Four strains were probably *Bacterium pneumosintes* or group 2. Three of these came from patients with influenza and one from a normal person (table 2). One of the influenza strains was isolated directly from a blood agar plate; the others grew first in the ascitic-kidney medium and were later plated on blood agar. The colonies were not visible to the unaided eye. With low power they could be seen plainly as smooth translucent droplets. Stained preparations made from these showed minute gram-negative rods. *Bacterium pneumosintes* and the group 2 organisms resemble each other so closely morphologically and culturally that I am unable to tell the difference between them on that basis. One of these strains could not be carried beyond the first culture generation; the others were carried through 3, 4 and 7 successive culture generations on blood agar; but at no time was there a sufficient quantity of the organisms obtained to attempt positive identification by agglutination tests, even had *Bacterium pneumosintes* antiserum been available for use.

The remaining 4 strains seemed to belong to 2 groups hitherto undescribed. In one group, including 2 strains, 1 from influenza and 1 from a cold, the gram-positive organisms occurred in definite clumps, and were so minute that no conclusion could be drawn as to their shape. The colonies were microscopic, round, raised, and transparent. Both of these strains were lost after the fourth culture generation on blood agar. The 2 strains of the other group, both from colds, have been kept for many months and seem to be identical, morphologically and culturally. Stained preparations show a small gram-negative coccus, always in pairs, and occasionally in chains of 4 to 10 cocci. As a rule they are neither flattened nor elongated. In the ascitic-kidney cultures the growth quickly settles on the sides and in the bottoms of the tubes. There has been no gas formation observed in these cultures. On blood agar plates plainly visible colonies are formed. These are round, smooth, shining, raised, and translucent. On crowded plates the growth sometimes becomes confluent. These strains are hemolytic when grown on rabbit blood agar, though very little hemolysis has been observed on plates made with sheep blood. These 2 strains have not been pathogenic for mice, guinea-pigs, or rabbits when inoculated intraperitoneally, or for rabbits when given intravenously.

The fact that these organisms were isolated from Berkefeld filtrates should not be interpreted as proof of their true filtrability. Only V filters were used, since the object was merely to hold back the common mouth organisms and to have conditions as favorable as possible for the small anaerobes to pass through. The factors influencing filtration are numerous and variable, and many bacteria can pass a Berkefeld V filter under some conditions.

SUMMARY

Nasopharyngeal washings from 26 persons, including 13 with colds, 5 with acute influenza and 8 normal persons, were examined by the technic of Olitsky and Gates. From 11 of these samples 17 strains of anaerobic microorganisms were isolated, 13 of which seem to belong to species previously described. Four strains resembled the gram-negative, gas-producing coccus found abundantly in normal mouths by Holman and Krock and by Hall and Howitt. Two were probably the large granular spirochete of sinus infections which was described by Tunncliffe. Seven were apparently the filtrable microorganisms reported by Olitsky and Gates: 2 of these seemed identical with their slender curved group 1, and 1 with the granular group 3. Four were probably either *Bacterium pneumosintes* or the group 2 which resembles it; 3 of these came from patients with influenza during March, 1926, and 1 from a normal person 2 months after the epidemic had subsided.

The remaining 4 strains seem to fall into 2 groups, hitherto undescribed. One of these groups was a minute gram-negative organism of indeterminate morphology. The other, a small gram-negative hemolytic diplococcus, is described briefly here.

A TOXIN-PRODUCING HEMOLYTIC STREPTOCOCCUS FROM SEPTICEMIA

I. PILOT AND R. E. WESTLUND

From the Departments of Pathology and Bacteriology, University of Illinois College of Medicine, and Northwestern Medical School, Chicago

Hemolytic streptococci from conditions other than scarlet fever are known to produce toxins. Strains from erysipelas¹ and from puerperal sepsis² produce toxins which have been distinguished from the toxin of scarlet fever streptococci when tested in the skin of normal persons. The present study concerns a toxin-producing hemolytic streptococcus which was isolated from the blood of a patient who died of septicemia following an infection of the finger.

A machinist, 64 years of age, entered the hospital complaining of an infected finger. His finger had been caught in the gear of a machine two weeks before but he continued work until he noticed marked swelling and tenderness. Hot applications were applied without improvement. The infection spread from the right index finger to the hand which, when incised, yielded a thin seropurulent exudate. The leukocyte count was 20,650, the temperature varied from 100 to 103 F. After palmar and dorsal incisions the temperature dropped to 99 F. for three days, then rose rapidly to 103 F. On the following day he had several chills and a temperature of 105 F. At this time a blood culture was made which yielded a profuse growth of streptococci. Mercurochrome (30 cc.) was given intravenously, but the patient died on the following day. At no time was a skin rash or sore throat evident. No history of contact with scarlet fever patients could be obtained from relatives.

The streptococcus formed typical biconvex colonies on blood agar with a clear zone of hemolysis 4 mm. in diameter. Lactose and maltose were fermented but mannitol and inulin were not affected. In the production of toxin the method of Dick and Dick³ was used.

The cultures were grown in an infusion broth containing 1% Witte's peptone, 0.3% Liebig's meat extract, 0.5% sodium chloride and 1% sterile defibrinated sheep blood. After incubation for 4 days the broth culture was filtered through a filter paper, then through a Berkefeld filter, and the sterility of the filtrate determined by cultures. Antitoxin was produced by the injection of gradually increasing doses (1 to 5 cc.) of the undiluted toxin subcutaneously in rabbits at intervals of 3 to 4 days. Sixteen injections were made followed by intravenous doses of 1 to 2 cc. of the same toxin. After 21 injections no appreciable amount of antitoxin was demonstrable, but after the 27th injection, the serum of a rabbit diluted 1:25 neutralized the toxin diluted 1:400.

Received for publication, May 8, 1927.

¹ Birkhaug, K. E.: J. Am. M. A., 1926, 86, p. 1411.

² Lash, A. E., and Kaplan, B.: Ibid., p. 1197.

³ Ibid., 1924, 83, p. 84.

The toxin diluted 1:2000 and injected intradermally produced a marked reaction: 0.2 cc. forming a zone of erythema 15 to 30 mm. in diameter. This amount of the 1:2000 dilution constituted one skin test dose as compared with the U. S. Hygienic Laboratory standard. Of 172 persons from 6 to 50 years of age, 53 or 30.8% gave positive reactions. Of these a group of 110 from 20 to 30 years of age, 43 or 39% reacted. Of 60 persons tested simultaneously with toxins from a scarlet fever streptococcus and from the septicemic strain, 13 reacted with the toxin of the septicemic strain and 10 of these with the toxin of the scarlet fever strain. On retesting with the toxin of another scarlet fever streptococcus (U. S. Hygienic Laboratory) the other three also gave positive tests. The remainder of the group was negative. Thirty-two convalescent scarlet fever patients who had passed the 20th day of the illness failed to react to the toxin of the septicemic strain.

In the neutralizing tests with antitoxin the method recommended by Dick and Dick⁴ was used. The rabbit antitoxic serum diluted 1:25 neutralized toxins of both the scarlet fever and the septicemic strains in dilutions of 1:400. Commercial scarlet fever antitoxin similarly neutralized both toxins in the indicated units per cc. (1:25,000).

In the preparation of agglutinating serum, rabbits were given intravenous injections. The first injection consisted of 1.5 cc. of a 24-hour ascitic dextrose broth culture previously heated to 60 C. for 30 minutes. No agglutinins were demonstrated on the seventh day. A second injection of 0.25 cc. of a living 24-hour culture was then administered. On the sixth day a titer of 1:340 was demonstrable. The amounts were increased until 1 cc. of the living culture was given at weekly intervals. After four doses the highest titer obtained was 1:640. The above technic was followed in accordance with Tunnicliff's⁵ demonstration that few doses are necessary as otherwise polyvalence develops. Gordon⁶ considers 1:400 a good titer for streptococci although 1:800 is possible.

For the agglutination tests the organisms were grown in beef heart infusion medium containing 1% dextrose, 1% peptone, 0.1% disodium hydrogen phosphate. Ascitic fluid was added in the proportion of 1:3. The final P_H of the medium was 7.6. A heavy growth of streptococci was obtained in this medium after incubation for 24 hours at room temperature. This agrees with the work by Shibley⁷ who reported more stable suspensions with organisms grown at 20 C. than at incubator temperature. The cultures were centrifugated and the organisms sus-

⁴ J. Am. M. A., 1925, 84, p. 802.

⁵ Ibid., 1926, 87, p. 625.

⁶ Brit. M. J., 1921, 1, p. 632.

⁷ J. Exper. Med., 1924, 39, p. 245.

pended in plain phosphate broth, P_H 7.6. The serum dilutions were made also with phosphate broth and the mixtures incubated for 2 to 2½ hours at 55 C. By this method satisfactory agglutination was obtained with 11 strains from various sources (nonscarlatinal) as well as with a scarlet fever strain. All strains including the homologous organism were agglutinated in the same dilution (1:640).

Absorption tests were made according to Tunncliffe's⁸ method. The cultures were grown in ascitic phosphate broth at 37 C. for 24 hours, centrifugated at high speed, the clear supernatant fluid was removed, and 1 cc. of the immune serum, diluted 1:10 with phosphate broth, was added to each tube containing the organisms. After incubation at 55 C. for 2 hours agglutination tests were made with the absorbed serums and the homologous coccus. Each of 20 strains of hemolytic streptococci from various sources as well as the scarlet fever streptococcus absorbed the agglutinins.

From our observations the septicemic strain resembles the hemolytic streptococci of scarlet fever in tests for toxin and for neutralization with antitoxic serums. Streptococci resembling strains from scarlet fever have been noted by Stevens and Dochez⁹ who isolated the organisms from acutely inflamed throats of patients who gave negative skin tests. Rosenow¹⁰ identified as scarlet fever streptococci by the precipitin test, strains from patients with tonsillitis and no rash, the pus of an infected finger of a scarlet fever patient and of an empyema complicating surgical scarlet fever and from milk before pasteurization. Williams¹¹ reports one strain from a wound, another from a case of endocarditis, six from excised tonsils, all of which produced toxin neutralizable by convalescent scarlet fever serum.

Gordon,⁶ Stevens and Dochez,⁹ Tunncliffe⁵ and others have shown specific agglutinations for the scarlet fever streptococcus. Our experience with the agglutination and absorption tests, however, failed to demonstrate the septicemic strain as one belonging to the scarlet fever group.

SUMMARY

A strain of *Streptococcus hemolyticus* isolated from the blood of a patient with septicemia produced a toxin which resembled the toxin of streptococci from scarlet fever in skin reactions and in neutralization tests with antitoxic serums. Agglutination reactions and absorption tests, however, failed to demonstrate a similar specific relationship.

⁸ Personal communication.

⁹ J. Am. M. A., 1926, 87, p. 2137.

¹⁰ Ibid., 1926, 86, p. 9.

¹¹ Am. J. Pub. Health, 1925, 15, p. 129.

EFFECT OF SURFACE TENSION ON THE GROWTH OF ESCHERICHIA COLI

WILLIAM R. ALBUS

From the Research Laboratories, Bureau of Dairy Industry, U. S. Department of Agriculture

The mechanism of the action of surface tension and surface-tension depressants in relation to biologic processes is as yet unexplained. Nevertheless, observed effects of variations in surface tension on bacteria and bacterial growth increase our knowledge, and the accumulation of such observations may ultimately reveal the mechanism of the action of surface tension on vital processes. Numerous papers have described the antiseptic properties of surface tension depressants and their influence on the efficiency of various antiseptics, but comparatively little work has been done with surface tension in relation to bacterial growth.

Larson, Cantwell, and Hartzell¹ studied the growth of several types of bacteria in mediums with reduced surface tension. They used castor oil soap as the depressant and found the growth of certain bacteria to be greatly influenced by the surface tension of the medium. Ayers, Rupp, and Johnson,² in their study of the streptococci, used various substances as depressants of surface tension. They found that the growth of various species of streptococci was suppressed in mediums at various levels of surface tension. Albus and Holm³ have shown that *Lactobacillus bulgaricus* is unable to grow in a medium depressed with sodium ricinoleate to a surface tension below 40 dynes, whereas *Lactobacillus acidophilus* grows well in the same medium depressed to 36 dynes. It has been quite generally accepted that the growth of *Escherichia coli*, as well as certain other common types of bacteria, is unaffected by a considerable lowering of the surface tension of the medium. Larson¹ and his associates found that *Escherichia coli* was able to grow in a medium in which the surface tension had been depressed to 32 dynes. Ayers, Rupp, and Johnson² report the growth of *E. coli* in a medium depressed to 35 dynes.

In a preliminary experiment it was observed that the cell population of a culture of *E. coli* grown for 18 hours in 1% peptone water was appreciably greater than that of a culture of the same organism grown for 18 hours in 1% peptone water to which a small amount of castor-oil soap had been added. Although cursory observations indicated a normal growth in both cultures, it was evident from the plate counts that the growth of the organism was affected in the first 18 hours by the presence in the medium of a small amount of castor-oil soap.

Received for publication, May 9, 1927.

¹ J. Infect. Dis., 1919, 25, p. 41.

² J. Infect. Dis., 1923, 33, p. 202.

³ J. Bact., 1926, 12, p. 13.

Bacterial growth curves demonstrate any disturbance in the normal functioning of the cells and therefore lend themselves most readily to studies relating to bacterial growth. Injurious agents invariably change the growth curve. The curve is of further significance in that it affords a quantitative method for the study of factors affecting the growth of bacteria. It is possible, therefore, to follow the development of a bacterial culture under a wide variety of conditions and to obtain curves from which a conception of the action of any disturbing agent can be formulated.

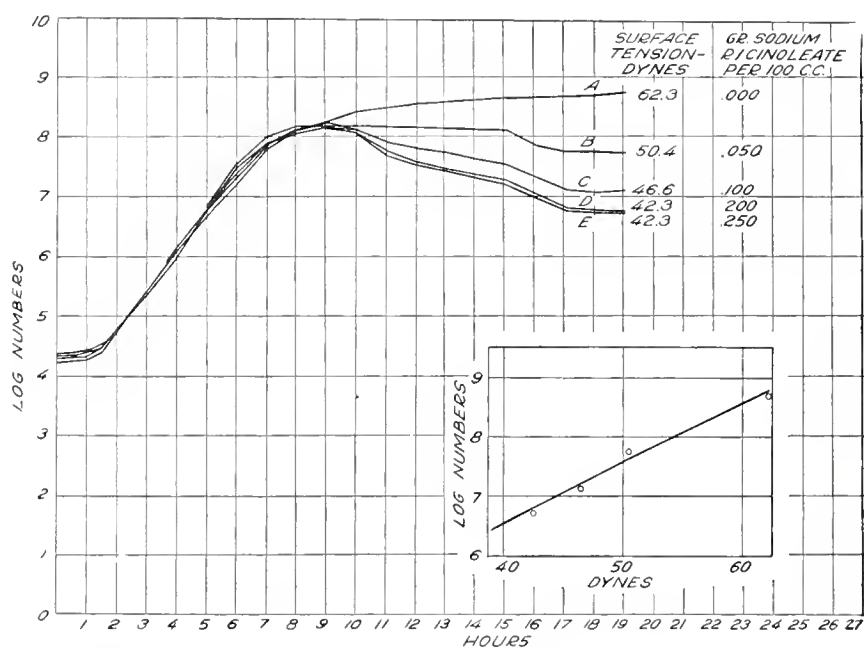


Fig. 1.—Relation of surface tension to growth of *Escherichia coli*.

Experiment.—The mediums used were put up in 100 cc. amounts; all contained 1% peptone and the amounts of pure sodium ricinoleate given in figure 1. The reaction of the mediums was not adjusted. The surface tension was determined, after autoclaving, by the drop-weight method. The organism used was a laboratory strain of *E. coli*. Inoculations were made from cultures 24 hours old at 37 C. Incubation was at 37 C. The plate counts were made on standard extract agar and were incubated for two days at 37 C. Duplicate plates were made for each dilution.

Figure 1 embodies the growth curves of five cultures of *E. coli* grown in 1% peptone water and in 1% peptone water containing the indicated amounts of pure sodium ricinoleate, and will serve to illustrate

the results obtained from a number of experiments. A sixth curve, showing the growth of this organism in 1% peptone water to which had been added 0.30 gm. of sodium ricinoleate per 100 cc. of medium, is not shown. This curve lay so close to the curves D and E that it was omitted to avoid confusion. The growth of six cultures was followed simultaneously. The cultures were inoculated two at a time at 15-minute intervals. The inoculum for the six cultures was from the same parent culture. Platings were made at intervals of one hour, two cultures being plated at the same time. It was found necessary to maintain the dilution blanks at a constant temperature (21 C.), and to complete the plating process as quickly as possible in order to avoid a marked mortality of the cells.

An inspection of the curves in the figure reveals the fact that in all of the cultures there was a normal growth up to a point between the 8th and 10th hours after inoculation, or just past the logarithmic growth phase. From this point to the 18th hour there was a marked mortality in those cultures growing in the mediums of lowered surface tension. The extent of the mortality increased with the degree to which the surface tension had been depressed.

It is striking that the mortality should appear after the logarithmic phase of growth has been passed for then the cells have begun to acquire the characteristics of old cells and are therefore more resistant to deleterious influences than when they are in the rapidly growing phase. The sensitivity of young cells has been demonstrated by Sherman and Albus.⁴ It is also to be noted from the curves that the mortality followed the surface tension rather than the concentration of sodium ricinoleate, a fact of possible significance in the mechanism of the action of surface tension on biological phenomena. This is more clearly shown in the lower right-hand portion of the figure.

The logarithms of the cell population of the 19-hour cultures in the mediums containing sodium ricinoleate are plotted as abscissas and the surface tension in dynes as ordinates. It will be seen that a straight line is thus obtained which illustrates the fact that the observed effect on the growth of *E. coli* follows the surface tension of the medium.

DISCUSSION

These results show that the growth of *E. coli* is affected by depressing the surface tension of the medium, a fact not heretofore evident because

⁴ J. Bact., 1923, 8, 2, p. 127.

the effect does not take place until after the organisms have passed through a normal logarithmic growth period. Previous investigators have accepted evidence of growth as a basis for their conclusions.

The full interpretation of these results cannot be attempted in our present state of knowledge. It is quite generally known that young bacterial cells are more susceptible to unfavorable influences than are mature cells. It is therefore most interesting to observe that, whatever the nature of the action on the cells may be, they show no susceptibility until after they have passed the period of rapid growth. They have then begun to take on the characteristics of mature cells and are therefore more resistant than during the preceding hours. This might be taken as very suggestive of the formation by the bacteria of some toxic substance were it not for other evidence which is contradictory to such an interpretation. Such an assumption would admit of the formation of a toxic substance from the soap, either as a metabolic product or as the result of the action on the soap of some product of metabolism. In either case the concentration of a toxic substance might reasonably be expected to increase with time and the concentration of the soap and would be indicated by a corresponding increase in the mortality of the cells. The curves do not indicate a concentration of toxic substance; furthermore, if the logarithms of the number of cells are plotted against surface tension a straight line is obtained. This indicates that the mortality follows the surface tension of the medium and not the concentration of the soap used as a depressant.

It has been shown frequently that the same effects are not observed at the same surface tension when different substances are used as depressants. This may possibly be due to "salt effects." Further studies embodying this subject are contemplated.

CONCLUSIONS

The growth of *E. coli* is affected by the surface tension of the medium as is shown by a reduction in the number of living cells in the culture. The cell mortality does not take place until after the culture has passed through a normal logarithmic growth period. While no explanation is offered concerning the nature of the action which brings about this cell mortality it is shown that it follows the surface tension of the medium rather than the concentration of the depressant.

THE EFFECT OF MOSAIC ON THE GLOBULIN OF POTATO

MAYME DVORAK

From the Experiment Station, North Dakota Agricultural College, Fargo

It is known that an intimate connection exists between the globulin of the animal and the etiologic agent in disease,¹ but so far as has come to the attention of the author, this effect seems to be largely quantitative. It would be difficult to demonstrate a like quantitative variation in the plant kingdom, but that a difference in globulin specificity may be induced seems to be clearly indicated by some recent results obtained in working with flax.² The globulin fractions from the seeds of flax plants differing only with reference to resistance or susceptibility to a definite disease, flax wilt, were shown to be highly specific and they may be differentiated by precipitin tests.

With the hope of demonstrating more clearly the effect of disease on the globulins of a host plant, precipitin tests were made of the globulins from healthy potato plants and from mosaic diseased plants. The plants were secured from tubers of the Triumph variety which had been planted in the greenhouse for seed certification * purposes. For several weeks the young growing plants were studied and carefully checked for the absence or presence of mosaic. All plants were grown under the same conditions, and dug up at the same time. The mosaic and healthy plants were each collected separately and washed free from dirt.

Antigens.—The usual methods employed to obtain fresh green plant extracts which give suitable globulin precipitates were tried and found unsatisfactory.³ Chinall⁴ in working with fresh green plant materials suggests a method for the extraction of the cell sap and the cytoplasmic contents of plants. This method applied to the fresh green potato plant material proved very satisfactory. From the extracts, it is possible to obtain globulin precipitates which when used as antigens, produce precipitins which are highly specific. In this work the tubers, the leaves and the stem including the root, were each separated and cut into coarse pieces. In order to plasmolize the cells the leaf portions were immersed in ether for one minute and the stem portions for two minutes. The materials were then agitated in the air to remove the ether. The leaf and stem portions were com-

Received for publication, May 7, 1927.

* These plants were secured from the State Seed Commissioner, Dean H. L. Bolley (plant pathologist), who also verified them.

¹ Wells, H. G.: Chemical Aspects of Immunity, 1925, p. 184.

² Nelson, C. I., and Dvorak, M.: No. Dakota Agric. College Exper. Sta. Bull. 202, 1926.

³ Osborne, T. B.: The Vegetable Proteins.

⁴ J. Biol. Chem., 1923, 55, p. 333.

bined, placed in a muslin bag and pressed in an ordinary fruit press. After the first extraction, sterile water (distilled) was added to the pulp, which was again pressed. Four such extractions were made, the last being almost colorless. The first extration was reddish brown in color. These extracts were combined and centrifuged. This constituted the "cell sap."

To the pulp was added a small amount of distilled water and the mass was ground in the Nixtamal mill. This macerated material was placed in a muslin bag and subjected to pressure, the process being repeated four times. The extract obtained was filtered and centrifuged. The resulting liquid, which was light green in color, constituted the "cytoplasmic extract."

The finely ground fibrous pulp which remained was covered with a 3% sodium chloride solution and allowed to soak over night. The extract pressed from this material and centrifuged was light straw colored. This was called "salt solution" extract.

These various extracts from the normal or healthy potato and the mosaic potato plants were each separately electrodialyzed against running distilled water. Each extract yielded globulin precipitates which were collected by centrifuging. To purify the globulins they were redissolved in physiologic salt solution made slightly alkaline by the addition of $\frac{N}{10}$ NaOH. They were reprecipitated by electrodialysis, and redissolved, for use as antigens.

The young tubers of each kind were separately ground in the Nixtamal mill. This ground pulp was placed in a small amount of 3% sodium chloride solution and allowed to stand over night. The liquid was expressed from this material and centrifuged. The globulins were precipitated by electrodialysis, redissolved and centrifuged. This solution of tuber globulin was used as antigen.

Early attempts to secure from green plant materials globulin fractions which could be satisfactorily used for antigen, did not meet with much success because of the failure to get the specific protein fraction concerned from the appropriate parts of the plant. The method described and used herein overcame many of the difficulties formerly encountered.

Injections.—Rabbits weighing approximately 2 Kg. were sensitized to the globulin solutions. Intraperitoneal injections of 1 cc. were given every five days until five injections in all had been given. Four days after the last injection approximately 15 cc. of blood was obtained from the large ear vein of the sensitized animal.

Precipitin Tests.—The globulin solutions used as antigens in these tests were centrifuged at 1,200 r. p. m. for five minutes to eliminate any large suspended globulin particles. The antiserum was used as the variable factor. While it is customary in most precipitin work with animal proteins to consider the titer of the precipitin as the least amount of antigen which will produce a precipitate with a given dilution of serum, in plant work in this laboratory it has been found best to vary the strength of the serum. In this work, serum dilutions varied from 1:10 to 1:200. Insofar as the amounts of material at hand permitted, direct and cross titrations were made between the homologous and heterologous units. All tests were incubated over night at 37 C. Twelve and 24 hour readings were recorded. The 24 hour readings were the most conclusive, and are shown in chart 1, graphs 1 to 32.

Discussions and Conclusions.—To facilitate a comparative study of the results obtained, all data have been collected and charted (chart 1). The plotted lines in each of the 32 graphs of the plate represent the

SERUMS

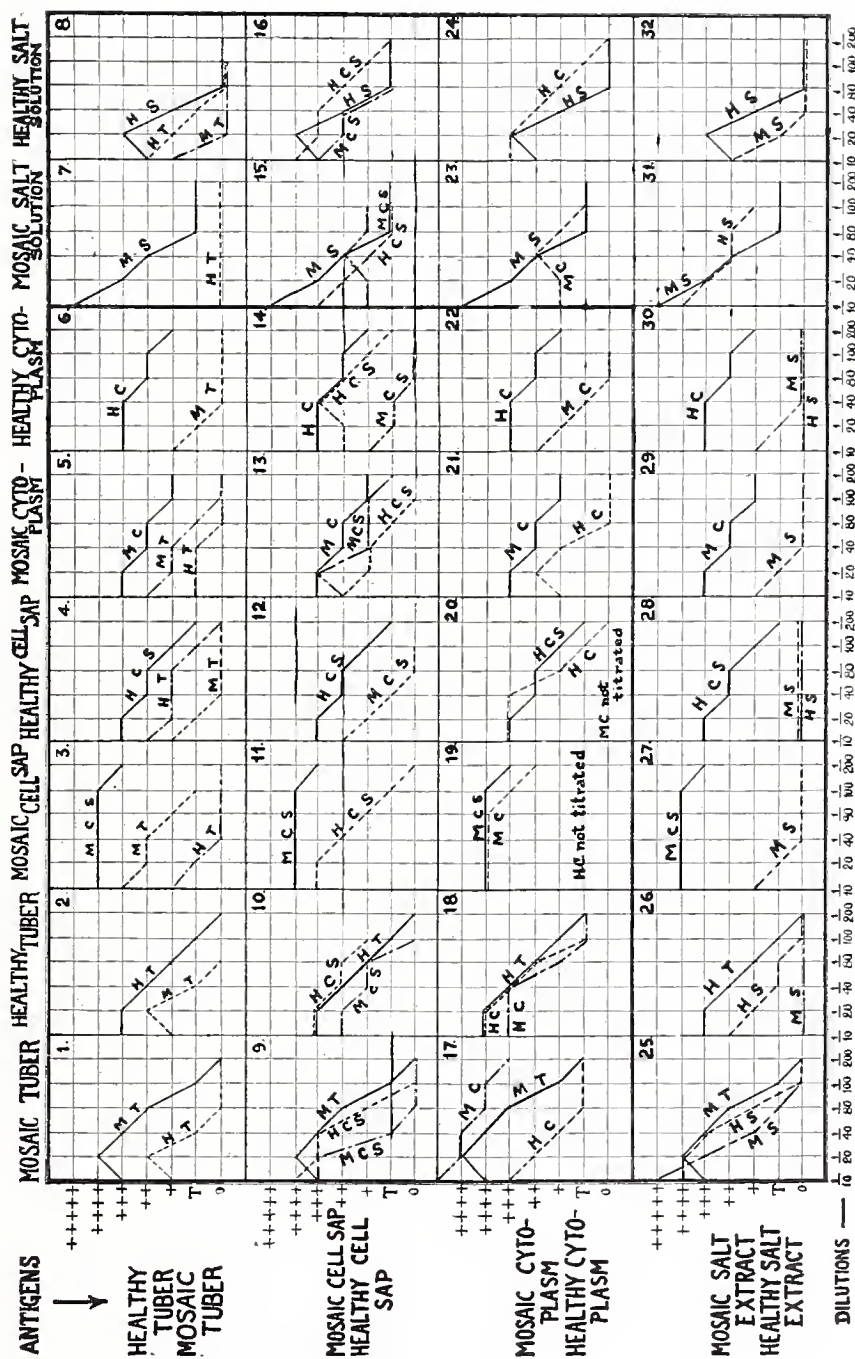


Chart 1.—Graphs of 24-hour readings of precipitin tests with plant globulins as indicated. MT indicates mosaic tuber; HT, healthy tuber; MCS, mosaic cell sap; HCS, healthy cell sap; MC, mosaic cytoplasm; HC, healthy cytoplasm; MS, mosaic salt extract; and HS, healthy salt extract.

results of titrations of the antigens listed at the left of the chart against the antiserums, which are listed at the top of the chart. To aid the reader in the interpretation of the chart, the respective antigens are lettered in each graph. The most significant graphs of the plate are indicated in the rectangular portion, which is inclosed by the inner heavy line. Within this portion are represented the results of titrations between the tuber, the cell sap and the cytoplasm, and their respective antiserums.

Graphs 1 to 6, 9 to 14, 17 to 22 of three horizontal columns of this rectangular division are each to be studied separately. From this study, the following four facts may be noted: the highest titer in each case, represented by the solid line (except in graphs 10 and 17) is obtained between the homologous antigen and antiserum; the lowest titer, represented by a broken line in each graph is obtained by titrating the heterologous antigen and antiserum; in each case where the results of a titration of a serum with a third antigen is plotted, the broken line representing the data is intermediate to the other two. This third antigen (except in graph 9) represents one of the globulin fractions of the homologous group; and in the exceptions noted above in graphs 10 and 17, it is a heterologous globulin fraction which yielded the greatest precipitin reaction.

A more detailed study of this rectangular section is made by comparing the six vertical columns in which, so far as material available made it possible, the various antigens are each titrated against the antiserums produced by globulins of the tuber, the cell sap and the cytoplasm of each the mosaic and healthy plants. The facts as revealed in this study reveal that greater specificity is shown in the titration of each antigen—mosaic tuber (except G17), healthy tuber (except G10), mosaic cell sap, healthy cell sap, mosaic cytoplasm and healthy cytoplasm—with the respective homologous antiserum.

In graph 17 it is the cytoplasmic fraction of the homologous group which is the most specific against the mosaic tuber antiserum, and in graph 10 it is the cell sap of the homologous group which is the most specific when titrated against the antiserum of the healthy tuber.

A comparison of the homologous fraction with the corresponding heterologous fraction titrated against the homologous antiserum (graphs 1 and 2, 11 and 12, 21 and 22) shows that the homologous fraction possesses a higher titer than the heterologous fraction. The only difference between the antigens is the disease factor.

The greatest difference between the two occurs in the higher dilutions 1 to 40 and 1 to 80. This difference is most marked in the comparison

of cell sap and cytoplasm of both the mosaic and healthy plants (graphs 21 and 22). In addition, it is further shown that with globulin fractions from the heterologous group, the greatest difference in precipitin reaction occurs in the titrations of the antigens and antisera of the cell sap and cytoplasm (graphs 3, 4, 5, 6, 14, 17). This indicates that the factors which are determinative of specificity lie principally in the cell sap and cytoplasm of their respective plants.

The lack of significant difference in the titrations of these two antigens against the same antisera (graphs 13, 19, 14, 20) would seem to indicate that the cell sap and cytoplasm possess the specific factor in common.

The results of titrations of the salt extract globulins against the various antisera are plotted in the lower horizontal column and in the two vertical columns to the extreme right of the chart. In these graphs (25, 26, 28, 30, 7, 8, 15, 16, 23, 24, 31, 32) it is noted that little variation occurs in the precipitin reactions of the salt extracts used as antigens. Graphs 27 and 29 do not represent complete cross titrations. The slight difference that is noted (graphs 15, 16, 24) may be due to globulin remnants of cell sap and cytoplasm left in the pulp material from which the salt extracts were made. Complete, or almost complete, lack of specificity is noted in graphs 27, 28, 29 and 30. The determinative factors of specificity were removed in the previous extracts of cell sap and cytoplasm. Little if any difference exists between these two salt extract globulins used as antigens with the respective antisera (graphs 31 and 32). This would indicate that they are very similar globulins.

For the purpose of getting a mathematical expression of averages, the following arbitrary numerical values have been assigned to the amounts of precipitates obtained in the reactions, 0=0, T=5, +=5, ++=20, etc. The numerical values for each, the homologous fractions, the homologous group fractions and the heterologous group fractions were added and averaged. The salt extract titrations have been omitted since they show little if any difference in specificity. The averages obtained are included in the following tabulation:

Dilutions	1:10	1:20	1:40	1:80	1:100	1:200
Homologous fraction	31.66	33.33	26.67	21.67	13.33	6.67
Homologous group fraction	25.87	22.32	16.67	13.47	7.99	2.97
Heterologous group fraction	15.70	12.57	7.84	2.57	0.40	0.00

These averages are graphically represented in chart 2. A study of this chart confirms the results represented graphically in chart 1, i. e., taken collectively: the homologous fraction is the most specific as shown

by the higher precipitin titer; the heterologous group fraction is the least specific as shown by its low titer; the homologous group fraction lies intermediate in its precipitin reaction, indicating its close relationship to the homologous fraction.

It may be claimed that the disease factor may do one of two things, it may introduce an independent globulin derived from the virus, in addition to the plant globulin, or it may alter the serologic character of the plant globulin. The foregoing data do not present a brief for either contention, but plainly leads to the conclusion that the disease factor has

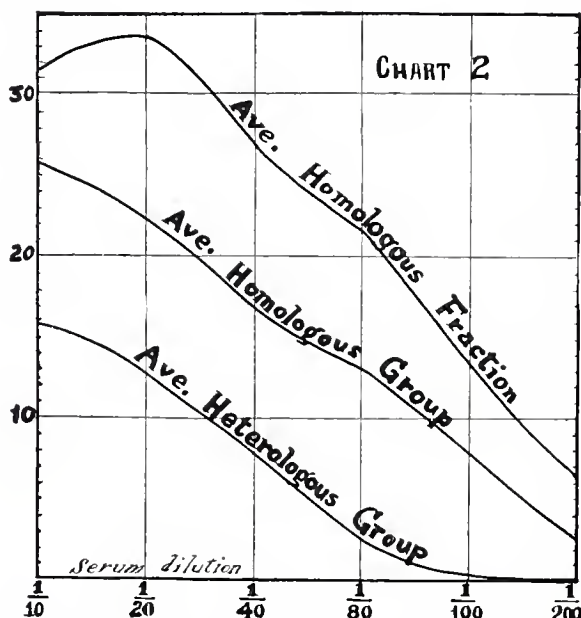


Chart 2.—The average amount of precipitate obtained with different dilutions of antisera with homologous fractions and with homologous and heterologous group fractions as indicated.

influenced the precipitability of the globulins of the cell sap and the cytoplasm of the potato plant. The principal points in the work are as follows:

Various plant extracts, consisting of cell sap, cytoplasm, immature tuber and a 3% sodium chloride solution were obtained from healthy and mosaic plants. These extracts yielded globulins which were highly specific when used as antigens.

In all cross titrations, with but two exceptions, the homologous antigens give the highest titer, the exceptions show that it is an antigen of the homologous group fraction that gives the highest precipitin titer, the heterologous antigens give the lowest titer, and homologous group antigens give a titer intermediate to the other two.

Titration results show that the tuber, the cell sap and cytoplasm antigens of both the mosaic and healthy plants gives the highest precipitin titer against their homologous antisera. Each is most specific against its homologous antiserum.

The results of titrating homologous cell sap and cytoplasm globulins against the antiserums of the same, would indicate that the specific factor is common to both of these fractions.

This factor possessed in common by both the cell sap and cytoplasm globulins against the antiserums of the same, would indicate that the specific factor is common to both of these fractions.

This factor possessed in common by both the cell sap and cytoplasm was evidently exhausted from the plant material before the salt extracts were obtained.

The comparative averages of the group studies show that the homologous fraction is the most specific (because it produces the highest precipitin average), the fractions of the homologous group are the less specific, while the fractions of the heterologous group are the least specific.

CONCLUSION

The conclusion to be derived from these findings is that the mosaic disease seems to affect the globulin fractions of the cell sap and cytoplasm of the potato plant, in such a manner as to change their precipitability by specific antiserums.

AGGLUTININ RESPONSE TO CERTAIN BRUCELLA ABORTUS BACTERINS

S. J. SCHILLING AND WM. L. BLEECKER

From the Laboratories of the Departments of Veterinary Science and Bacteriology of the Agricultural Experiment Station, Fayetteville, Arkansas

In most of the experimental work on the immunization of cattle against *Brucella abortus* vaccines or bacterins have been used as immunizing agents. Neither of these products has proved to induce a high degree of immunity in the treated animals. Bacterins are generally regarded today as having very low antigenic qualities. That this is not always the case can best be ascertained by a tabular comparison of the results of various investigators who used both bacterins and vaccines (table 1). It is considered desirable to call attention to the evidence that bacterins may reduce the abortion rate as satisfactorily as vaccines, and since bacterins do not possess some of the inherent undesirable qualities of vaccines they may merit further study than has been directed toward them.

The means which have been used in attempting to produce immunity against infectious abortion in cattle include, first, vaccination with live cultures: (a) virulent live cultures without the use of immune serum, (b) virulent live cultures with the use of immune serum, and (c) live cultures of reduced virulence; second, treatment with bacterin: (a) bacterin alone, (b) bacterin plus serum; and third, treatment with bacterin followed by vaccination. The investigations on *Br. abortus* immune serum, used either alone or in conjunction with vaccines or bacterins, and investigations on attenuated vaccines and bacterins followed by vaccines have up to this time been very limited. Bacterins and vaccines made from virulent cultures have been used more frequently in experimental work; reference will be made to the work related to the various phases of investigation presented.

It has been pointed out by research workers that the value of immunizing agents against infectious abortion can not be judged solely by the termination of subsequent pregnancies, but that other factors such as the incidence of sterility, the retention of fetal membranes, the viability of the full term fetus, and the incidence of white scours in the calves should be correlated with the records of normal parturitions and abortions occurring among treated and control animals. Careful

records of the bacteriologic and serologic findings in all cases are also needed in evaluating the results of experiments in order that the results may be properly interpreted. Such supplementary data help in determining whether the observed symptoms of abortion disease occurring among treated animals or among controls are in fact due to *Br. abortus* or due to some other cause; they also enable us to locate evidence of uterine, mammary, or lymphatic infection, i.e., evidence of

TABLE 1

A COMPARISON OF THE ABORTION RATES IN ANIMALS TREATED WITH *BR. ABORTUS* VACCINE AND BACTERIN AND IN UNTREATED ANIMALS

Investigator	Immunizing Agent Used	Exposure of Treated Animals and Controls	Treated Animals			Controls			Remarks
			Total	No. Aborted	% Aborted	Total	No. Aborted	% Aborted	
Stockman ¹	Live Culture	Infected herd and premises	493	32	6.49	432	101	23.39	All ages
			65	4	6.10	47	7	14.80	Heifers
	Bacterin	Infected herd and premises	110	23	20.90	432	101	24.39	All ages
Gminder ²	Live Culture	Infected herd and premises	220	15	6.90	609	128	21.0	All ages
	Bacterin	Infected herd and premises	370	69	18.6	609	128	21.0	All ages pregnant and non-pregnant
Schroeder ³	Live Culture	Infected herd and premises	617	81	13.12	294	52	17.7	All ages, same herds two successive years
			311	32	10.29	142	21	14.9	
	Bacterin	Feeding infective material	11	1	9.90	8	7	87.5	All ages
		Feeding infective material	4	2	50.00	8	7	87.5	All ages
	Live Culture	Infected herd and premises	53	9	16.70	134	34	25.1	All ages
Smith and Little ⁴	Bacterin	Infected herd and premises	9	0	0.00	38	15	41.6	Heifers
			34	5	14.70	38	15	41.6	Heifers

the carrier state, in animals which may nevertheless carry their calves full term and give no visible evidence of abortion disease. The importance of such complete studies for a proper interpretation of data is not to be minimized; in the earlier studies much of this information was not obtained and is thus not available today. The amount of work involved in securing complete data even in a small experimental herd is considerable, and when experimental work is conducted in the field on large

¹ J. Comp. Path. & Ther., 1914, 27, p. 237.

² Berlin Tierarztl. Wchnschr., 1919, 35, p. 163.

³ J. Am. Vet. M. A., 1922, 60, p. 542.

⁴ Monograph, Rockefeller Inst. for M. Research, 1923.

numbers of animals on widely distributed premises, it tends to become prohibitive; thus even in the later work presented the corollary information is sometimes wanting. These factors are not evaluated in most of the data presented in table 1, but the large numbers of animals involved in the experiments reported help in a degree to overcome the omission. The table, then, has significance in presenting a comparison, as between treated and control animals, of the frequency of occurrence of the most prominent of the pathologic consequences, namely, premature expulsion of the fetus. The suppression of abortions will serve to some extent as an index to the suppression of other manifestations of the disease and of the disease itself.

An examination of the table reveals in all cases a smaller number of abortions among treated animals than among untreated controls. This is true of the bacterin treated as well as of the vaccinated animals. However, in the work of Stockman,¹ Gminder,² and Schroeder,³ the efficacy of vaccines appears to be greater than that of bacterins in reducing the number of abortions. A similar conclusion was reached by Fitch and Boyd⁵ in work which is not represented in this table. On the other hand in later work Gminder⁶ found a reduction from 25.21% to 15.15% in the abortion rate among vaccinated animals, and from 18.5% to 13.02% among bacterin treated animals, while among the controls the abortion rate increased from 16.13% to 22.68%. These findings indicate that bacterins may be as efficacious as vaccines. The work of Smith and Little⁴ (table 1) also gave results indicating that bacterins may have as high an immunizing value as vaccines. These investigators suggest that it might be of value to increase the number of injections of bacterin to six or eight, in order to maintain the immunity over a larger portion of the gestation period.

In a number of investigations on immunizing agents vaccines only were used on the treated animals. Under such experimental conditions Hadley⁷ in one experiment obtained a reduction of the abortion rate from 31% to 14% and in another trial a reduction from 33% to 22%. Again Lubbelhusen, Fitch and Boyd⁸ found an abortion rate in a group of vaccinated animals of 19% while among untreated controls the rate was 28.7%. Admittedly a valid comparison could here more easily be made if bacterins had also been used in these trials. However in these instances the results following vaccine treatment are on the whole inferior to those obtained by Smith and Little in their trials with bacterins and this indicates that the view held in some quarters that vaccines possess superior immunizing qualities over bacterins may require qualification.

The results of experiments in which small animals were used are of interest. According to Hagan,⁹ Ascoli used a bacterin in which the organisms were killed by ether for the immunization of guinea-pigs, without preventing infection by subsequent inoculation of virulent *Br. abortus* organisms. Stafseth¹⁰ also working on guinea-pigs found that no immunity was produced by the use of heat killed culture used as a bacterin. Hagan⁹ has, however, pointed out that Stafseth used

⁵ J. Am. Vet. M. A., 1924, 65, p. 407.

⁶ Arb. Reichsgesundtsampt., 1920, 52, p. 375.

⁷ J. Am. Vet. M. A., 1921, 60, p. 26.

⁸ Minnesota Agr. Exper. Sta. Bull. 43, 1926.

⁹ J. Exper. Med., 1922, 36, p. 711.

¹⁰ Am. Vet. Review, 1913, 44, p. 307.

extremely large doses of virulent organisms in testing the immunity of his animals, and in his own work found that while guinea-pigs cannot be rendered entirely immune by injections of bacterin, that the progress of the inoculation disease can be considerably delayed.

The variation in the results obtained by various investigators suggest that a difference in the preparation of bacterins, a difference in the antigenic quality of the strains of *Br. abortus* used, or a difference in the conditions under which the work was undertaken must have existed. The experimental work of this paper relates to the use of a modified technic in the production of bacterins; and the administration of massive and repeated doses of bacterins compared with the vaccine treatment. The study undertakes to determine whether modifications of the antigen are introduced when cultures are killed in the process of making a bacterin. The usual means of killing cultures in making *Br. abortus* bacterins is the application of heat. No uniform method, however, has been adopted as to the temperature employed or the length of time the organisms are exposed to that temperature. It was considered that this might account at least in part for the discrepancy which may be noted between the results of various workers who have tested the immunizing value of bacterins. It is conceivable that the means used in killing the cultures causes a chemical alteration of the antigen which impairs its efficacy.

Experimental Procedure.—By a series of tests it was found that the addition of 0.5 per cent phenol to a suspension of *Br. abortus* which is then held at 35 C. for eighteen hours results in the death of all organisms therein contained. It was also found that a temperature of 55 C. would kill all of the organisms of a suspension within thirty minutes. Bacterins prepared by both of these methods and others prepared by the addition of 2 per cent tricresol, of 1 per cent ether, and of 1 per cent chloroform and holding at room temperature for twenty-four hours were injected into rabbits and the agglutination titer of the animals so treated was compared with that of rabbits injected with one large dose of living, virulent organisms. In performing the agglutination test the method used conformed to the recommendations of the committee of the U. S. Live Stock Sanitary Association on the standardization of that test. The antigen was a suspension of *Br. abortus*, grown on agar, washed off with phenolized salt solution, and diluted with the same to a density of tube one, McFarland nephelometer. Incubation at 37° C. for forty-eight hours was employed before readings were obtained. The work with minor modifications was repeated three times in order to check results. The animals used in the successive tests are indicated as series 1, 2 and 3, respectively.

Series 1.—A single strain of *Br. abortus* was used for the preparation of the vaccine and the various bacterins. This strain had recently been isolated from an aborting cow, and the organisms grew luxuriantly on ordinary pork agar. In making vaccine and the different bacterins, the cultures were grown on large agar slants for 48 hours and washed off with physiologic salt solution. The suspension was diluted to the density of a 24 hour broth culture of *Eberthella typhi*. Portions of this suspension were then treated by one of the methods given to kill the organisms and a portion was retained without treatment to serve as a vaccine. Thus the various bacterins and the vaccine had approximately equal bacterial content. After their preparation each bacterin was cultured so that we might be certain that all organisms had been killed. These preparations were used promptly after being prepared and the vaccine was cultured just prior to administration in order to make certain that it contained living organisms.

TABLE 2
VACCINE AND BACTERIN TREATMENT OF RABBITS OF SERIES 1 AND THE RESULTANT AGGLUTINATION TITERS

Antigen Used	Dates of Injections	Amount Ce.	Rabbit Number	Agglutination Titer (Highest reacting dilution)			Remarks
				1st Month	2nd Month	4th Month	
Br. abortus living culture	2/ 9/24	5	1	10240	10240	10240	
			2	10240	10240	6400	
Br. abortus killed by 0.5% phenol at 35 C.	2/ 9/24	2	3	3200	2560	2560	
	2/16/24	3	4	800	1260	5120	
	2/23/24	4					
Br. abortus killed by heat at 55 C.	2/ 9/24	2	5	5120	5120	5120	
	2/16/24	3	6	5120	10240	6400	
	2/23/24	4					
Br. abortus killed by 2% tricesol	2/ 9/24	2	7	No test obtained			Died Feb. 28, 1924
	2/16/24	3	8	160	640	640	
	2/23/24	4					
Br. abortus killed by 1% ether	2/ 9/24	2	9	10240	10240	1280	Died Mar. 1, 1924
	2/16/24	3	10	No test obtained			
	2/23/24	4					
Br. abortus killed by 1% chloroform	2/ 9/24	2	11	5120	6400	3200	
	2/16/24	3	12	6400	6400	6400	
	2/23/24	4					

Twelve rabbits were used. Rabbits 1 and 2 were vaccinated each receiving subcutaneously 5 cc. of a suspension of living, virulent organisms. Rabbits 3 and 4 were injected subcutaneously with three injections of a suspension of *Br. abortus* killed by the addition of 0.5% phenol and holding at 35 C. for 18 hours. Rabbits 5 and 6 received three subcutaneous injections of the *Br. abortus* suspension killed by heating on a water bath at a temperature of 55 C. for 30 minutes. Rabbits 7 and 8 received three subcutaneous injections of the suspension killed by the addition of 2% tricesol. Rabbits 9 and 10 received three subcutaneous injections of the suspension of *Br. abortus* killed by the addition of 1% of ether. Rabbits 11 and 12 received three similar injections of the suspension of the organisms killed by 1% chloroform.

The vaccine was administered to rabbits 1 and 2 on the same date that the other rabbits received the first injection of bacterin. One month later the blood serum of each rabbit was tested by means of the agglutination test; this test was repeated at two and four months. Since rabbits 3 to 12 received three separate injections of bacterin, the first agglutination test was conducted only two weeks after the last injection.

A number of agglutination tests were made on the blood of untreated rabbits in order to determine if agglutinins for *Br. abortus* were normally present. In

no instance did agglutination occur in as high a dilution as 1:20. It was also found that in no case did agglutination occur where the blood serum of the treated rabbits was tested against the strain used for immunization. However, the blood serum of the treated rabbits readily agglutinated a mixture of five other strains of *Br. abortus*. For all tests here reported, then, we used a suspension of a mixture of these five strains as the antigen.

Series 2.—Five newly isolated strains of *Br. abortus* were selected for preparation of the antigen. After growing each strain separately for 48 hours on pork agar slants and suspending the resultant cultures in salt solution, the suspensions were mixed in approximately equal proportions, and the bacterins prepared from this mixture of five strains. The procedures and precautions used

TABLE 3
VACCINE AND BACTERIN TREATMENT OF RABBITS OF SERIES 3 AND THE RESULTANT AGGLUTINATION TITERS

Antigen Used	Dates of Injection	Amount, Ce.	Rabbit Number	Agglutination Titrers (Highest Reacting Dilutions)				Remarks
				1st Month	2nd Month	4th Month	6th Month	
Br. abortus living culture	2/ 3/25	2	13	6400	20480	1600	1280	Died
	2/10/25	4	14	6400	12800	10240	No test	
	2/21/25	6						
	2/27/25	8						
Br. abortus killed by 0.5% phenol	2/ 3/25	2	15	6400	6400	10240	1280	
	2/10/25	4	16	6400	6400	10240	1280	
	2/21/25	6						
	2/27/25	8						
Br. abortus killed at 55 C.	2/ 3/25	2	17	5120	5120	800	480	Died
	2/10/25	4	18		No test obtained			
	2/21/25	6						
	2/27/25	8						
Br. abortus killed by 2% tri-cresol	2/ 3/25	2	19	5120	6400	800	640	Died
	2/10/25	4	20		No test obtained			
	2/21/25	6						
	2/27/25	8						
Br. abortus killed by 1% ether	2/ 3/25	2	21	6400	6400	2560	640	Died
	2/10/25	4	22		No test obtained			
	2/21/25	6						
	2/27/25	8						
Br. abortus killed by 1% chloroform	2/ 3/25	2	23	320	320	160	160	
	2/10/25	4	24	1280	1280	160	80	
	2/21/25	6						
	2/27/25	8						
Br. abortus living culture	2/ 3/25	5	25	6400	6400	640	640	
			26	6400	6400	320	80	

in preparing the vaccine and bacterins in the foregoing experimental work were again used. Two rabbits of this series were given four injections of living organisms, the quantities administered being the same as those given of the several bacterins. In this series the size of the doses was quite largely increased and four injections of bacterin, in place of three, were administered. Agglutination tests following the method outlined for the rabbits of series 1 were made at one, two, four, and six months.

Table 3 gives the kind of antigen and the doses each animal received as well as the agglutination titer of each animal at the time of test.

Series 3.—On the basis of results from the foregoing work it became apparent that a larger number of animals was necessary in order to compensate for the loss of experimental animals before completion of the trials and to compensate for individual variations in response to treatment. Accordingly bacterins and vaccines were prepared and employed as before; however, ten rabbits were used in each group; the group receiving three injections of living

culture, however, was omitted. The cultures used in preparing vaccine and bacterins for series 3 were the same as those used in series 2. Agglutination tests were made at one, two, and three months after treatment. Table 4 presents the necessary data regarding administration of treatment and response in agglutination titer of the sixty animals employed.

At the time of preparation of vaccine and bacterins for immunization of rabbits used in series 3 one of the strains of *Br. abortus* which had been employed in making the tests on series 2 was presenting atypical growths on culture medium; it was therefore omitted and only four of the strains formerly used were employed. Moreover, when agglutination tests were applied to the serums of rabbits of series 3, using an antigen containing the bodies of equal quantities of each of the four strains employed in making up the vaccine and bacterins, incomplete agglutinations only were observed. To locate the difficulty each strain of the organism was used separately to produce antigen and these antigens were used in the agglutinating system against a number of selected rabbit sera. It was thus found that two of the strains of *Br. abortus* used in immunizing rabbits of series 3 had lost their agglutinability. The antigen used in making the tests in series 3 was then made up with the two agglutinable strains.

It is to be noted further that the strain of *Br. abortus* used for treating animals of series 1 was a newly isolated strain pathogenic for guinea-pigs. The same was true of the five strains used in treating animals of series 2. At the time of conducting the tests on animals of series 3 it was found by inoculating guinea-pigs in duplicate with the separate strains of *Br. abortus* used in that test, and by careful necropsy and culture of the guinea-pigs four weeks later that the organisms used no longer were virulent.

Discussion.—In evaluating the immunologic response secured by treatment with vaccine and bacterins the determination of the agglutination titer was used. Experimental evidence obtained by investigators¹¹ relating to the validity of results secured by such procedure indicates that there is no direct parallelism in other infectious diseases between agglutination titer and bactericidal value or immunologic value of serums when such are tested for protective action in conferring passive immunity.

Stickdorn,¹² in two recent communications bases objections to the use of the agglutination or of the complement fixation titers of treated animals in determining the degree of resistance acquired on the grounds that in his experience after long continued treatment of animals with bacterins, the agglutination titer falls, whereas there may simultaneously have occurred an actual fortification of resistance to infection. Findings of this character serve to emphasize the fact that the accepted theories of immunity based largely on Ehrlich's concepts do not afford explanation for some of the phenomena encountered. For there is no logical explanation advanced for circumstances under which the antibody

¹¹ R. Paltauf in Kolle u. Wassermann; Handbuch der pathog. Mikroorganismen, 1913, 2, p. 509.

¹² Abstr. in Jahrsbr. Leist. Geb. Vet.-Med., 1924, p. 120.

TABLE 4
VACCINE AND BACTERIN TREATMENT OF SERIES 3 RABBITS AND RESULTANT
AGGLUTINATION TITERS

Antigen Used	Dates of Injections	Amount, Cc.	Rabbit Num- ber	Agglutination Titers			Remarks
				1st Month	2nd Month	3rd Month	
Br. abortus live culture	12/11/26	5	27	1280	—	—	Died 1/15/27
			28	160	40	640	
			29	1280	320	160	
			30	640	320	320	
			31	640	—20	—20*	
			32	3200	320	—20	
			33	20	320	—20	Died 1/20/27
			34	640	—	—	
			35	1600	160	80	
			36	1280	320	—	Died
			37	640	80	160	
			38	320	160	320	
Br. abortus killed by heat — 60 C. for 1 hour	12/ 4/26 12/11/26 12/20/26	2 3 4	39	—	—	—	Died 1/5/27
			40	1600	800	160	
			41	—20	40	—20	
			42	320	80	80	
			43	—20	80	—20	
			44	5120	80	40	
			45	320	—20	—	Died
			46	1600	3200	1600	
			47	320	—	—	
			48	20	160	—20	Died 2/16/27
Br. abortus killed with 0.5% phenol 35 C., 18 hours	12/ 4/26 12/11/26 12/20/26	2 3 4	49	80	—20	—20	
			50	20	320	80	
			51	640	80	40	
			52	2860	160	—20	
			53	20	160	80	
			54	—	—	—	
			55	1600	320	—20	Died 1/1/27
			56	1600	160	80	
			57	640	40	40	
			58	—20	160	80	
Br. abortus killed with 0.5% triere- sol, 18 hours at 35 C.	12/ 4/26 12/11/26 12/20/26	2 3 4	59	—20	20	—20	
			60	1600	20	—20	
			61	1600	—20	40	
			62	1600	20	20	
			63	80	20	—20	
			64	800	—	—	
			65	320	160	320	Died 2/1/27
			66	640	—20	—20	
			67	20	80	20	
			68	40	—20	—20	
Br. abortus killed with 1% chloroform	12/ 4/26 12/11/26 12/20/26	2 3 4	69	80	—20	—	
			70	160	800	—	
			71	—20	—20	—20	
			72	—20	40	40	
			73	—20	—20	—20	
			74	—20	—20	—20	
			75	D	—	—	Died 12/22/26
			76	—20	—20	320	
			77	1600	80	80	
			78	1600	40	40	
Br. abortus killed with 2% toluol	12/ 4/26 12/11/26 12/20/26	2 3 4	79	3200	—	—	
			80	1280	40	40	
			81	160	80	—20	
			82	1600	320	—20	
			83	—20	—	—	
			84	640	80	—20	
			85	2580	1280	3200	Died 2/5/27
			86	3200	640	40	

* The —20 means that the blood serum did not agglutinate in a dilution of 1 part with 19 parts of Br. abortus suspension which was the lowest dilution used.

production in response to a complex of antigenic substances such as are contained in a bacterial suspension may become selective. Either our theories of immunity are in need of revision or amplification or some of the experimental work bearing on this point was improperly controlled or interpreted.

Müller¹² in recent experimental work reported in two papers, and Brauneck¹² in another recent investigation also compared a number of agents used for immunization against infectious abortion using the agglutination and complement fixation titers as criteria for immunologic response. Both of these investigators believe to have demonstrated also that immunization of the animal tends to parallel agglutinin and immune amboceptor production to a significant degree. It may be anticipated that such a parallelism may not be absolute. Moreover, during long continued treatment of animals with bacterins, as Stickdorn reports, it is possible that this relationship may disappear. Still, in the early stages of a period of immunization a study of relative amounts of agglutinin produced as between differently treated animals may give a valuable index as to the entire complex of antigenic response.

Before undertaking any comparisons between the results obtained in this experiment on vaccine or bacterin treated rabbits, or between rabbits treated with different types of bacterin, we must make note of the wide variability of individual animals in antigenic response. For an examination of the tabulated data reveals that in many instances animals subjected to identical treatment differed widely among each other in agglutinins produced. A striking example is afforded by rabbits 43 and 44 in series 3, each of which animals had received similar quantities of heat-killed bacterin, yet one month later the former presented an agglutination titer of less than 1:20 while on the latter a value of 1:5,200 was obtained. Numerous instances of variability of this character are to be found in the data. This indicates the caution that must be observed in drawing conclusions from the results of experimental work of this nature. It also indicates one reason for the variability between the results of different investigators working on large animals and between the results of the same investigator reporting on different groups of animals.

The most important difference in agglutinin production is to be observed when the rabbits of series 1 and 2, comprising a total of twenty-six, are compared with those of series 3, where sixty animals were dealt with. The agglutination titers of the former composite group showed an unmistakable trend to run much higher than those of the

latter. Moreover, there was a significantly greater persistence of agglutinin titer in rabbits of series 1 and 2. These observations must be correlated with the difference in treatment of the groups of animals as a whole. The conditions of the several experiments were comparable except that rabbits of series 1 and 2 were treated with newly isolated virulent strains of *Br. abortus* while those of series 3 were treated with strains which by animal inoculation were demonstrated to have lost their virulence.

The four strains of *Br. abortus* used in series 3 had formerly been used in the antigen for series 2, thus, the question of origin of the strain does not enter into the consideration. Hence the modification of the strains in a year of laboratory cultivation by virtue of which their antigenic qualities had become dissipated is the factor responsible for this important difference. These results are entirely in accord with known facts regarding the variability of bacteria and the concomitant fluctuation in their antigenic properties as so recently correlated by Hadley.¹³

Since the antigenic value of the cultures from the standpoint of agglutinin production had shown such a marked decrease it is to be expected that their antigenic value in terms of capacity to induce a state of fortified resistance to infection in treated animals had also decreased. And if within a small group of strains of *Br. abortus* used in one laboratory fluctuations of biological character may occur so as to produce the divergent results here presented it raises the question whether herein may not be found another of the principal reasons for variability as between the results of other investigators. That significant antigenic dissimilarities existed as between the strains of the organism in question used by those investigators can not well be doubted. Thus, it appears that studies on the absolute or relative immunizing value of vaccines and bacterins made from cultures of *Br. abortus* must be so designed as to take into account the maintenance of the strains used at a comparable level of antigenic potency.

Moreover, the fact that the strain of *Br. abortus* used in treating animals of series 1 and that two of the strains used in treating animals of series 3 completely lost their agglutinability during the conduct of this work serves further to emphasize the variability of this organism in its immune body relationships and to suggest that all our concepts regarding the immunizing value of vaccines and bacterins in dealing with infectious abortion of cattle are in need of guarded interpretation.

¹³ J. Infect. Dis., 1927, 40, p. 1.

The tabulated results taking all animals into consideration show no distinct superiority of any one type of bacterin over the others as measured by the agglutination method. The results on vaccines as compared with bacterins show these to be of so nearly equal efficacy that we would not be warranted in holding one to be superior to the other. The differences in agglutination titer between groups of similarly treated animals do not hold constant when results on each separate series of animals are taken into consideration and they must be ascribed to be due largely to differences in individual response. What has been said in the foregoing statement holds also in respect to the relative persistence of agglutination titer as following different types of treatment. It is to be expected, however, that if following the administration of a virulent live culture the carrier state had been induced that in such a chronic infection a far greater persistence would follow.

Rabbits 25 and 26 of series 2 which received four injections of living culture attained for a short period a higher agglutination titer than other rabbits receiving four treatments with bacterin. This apparent indication of superiority of that type of treatment fails to be noted when the agglutination titers for rabbits 25 and 26 are compared with those for rabbits 15 and 16 in the later tests.

CONCLUSION

Several types of *Br. abortus* bacterins and vaccines were tested for antigenic efficacy in rabbits. The results were determined by the agglutination titers of the rabbit serum at selected periods. From three separate trials the following points were determined.

Marked individual variations in antibody production in the treated animals were found. The data suggest that in many animals *Br. abortus* vaccine or bacterin will induce no immunity whatever.

The loss of antigenic qualities of *Br. abortus* during cultivation on laboratory mediums was marked: a fact undoubtedly related to the variability of results already reported by investigators with large animals.

Treatment with live cultures did not uniformly induce a higher agglutination titer than treatment with bacterin. No uniform superiority appeared in any of the different types of bacterins in inducing high agglutination titer. The results do not indicate that the use of live cultures or of bacterins yields significant differences in the persistence of the immunity.

THE KAHN TEST IN EXPERIMENTAL SYPHILIS

JOHN E. WALKER

*From the Department of Clinical Pathology and Preventive Medicine, Army Medical School,
Washington, D. C.*

The Kahn test is a distinct technical advance in the serum diagnosis of syphilis. It is also evident that it is displacing the Wassermann test in an increasing number of laboratories. These considerations seem to render of interest a few observations that have been made of the reaction in normal and in syphilitic rabbits.

Technic.—The routine test was performed according to the technic prescribed by Kahn.¹ The serums giving + + + + reactions were further tested by the quantitative procedure. In the quantitative procedure standard antigen (exactly the same as in the routine test) was used. The concentration of the serums was as follows: 1:1, 1:5, 1:10, 1:20, 1:40, 1:80. If we combine the results of the routine and of the quantitative test we get the following series of symbols representing reactions of increasing strength: 0 (no reaction), \pm , +, + +, + + +, and + + + + with the strength indicated as 4, 20, 40, 80, 160, or 320 units. The Nichols strain of *Treponema pallidum* was used in the syphilitic animals.

Results with Normal Animals.—The serums of 29 normal male rabbits have been tested. The results in normal rabbit serum, however, are of interest only by comparison with results with normal human serums, since the test is proposed by its author as an aid in the diagnosis of syphilis in human beings, and not in rabbits. Normal persons for controls for an investigation of this kind are not easy to obtain. Certainly hospital patients will not answer. Nor will a group of supposedly normal persons chosen at random answer, since estimates of the prevalence of syphilis in the general population vary from 5% to 10%. However, during the past two years I have tested a group of 400 West Point candidates and student nurses, young adults from 17 to 25 years of age. A low rate of syphilis among them can safely be assumed, partly on account of their social status, but principally on account of their youth. Clinical evidence of syphilis had also been excluded in these persons by physical examination. The results with the normal

Received for publication, May 13, 1927.

¹ The Serum Diagnosis of Syphilis by Precipitation, 1925.

rabbit and human serums are shown in table 1. It is evident that there is a pronounced difference in the serologic condition of many rabbits as compared to human beings. An appreciable proportion of the rabbits have reactions as strong as + + + +. Only 36% of the rabbits were negative, while 99.8% of the humans were negative.

TABLE 1
RESULTS OF THE KAHN TEST IN 29 NORMAL RABBITS AND 400 PRESUMABLY NORMAL HUMANS

	Total Number Tested	Result of Tests							
		0	+	++	+++	++++			
						4 Units	20 Units	40 Units	80 and Over
Rabbits.....	29	11	5	4	5	2	1	1	0
Humans.....	400	399	0	1	0	0	0	0	0

0 = no reaction, and + to ++++ = reactions of increasing strength with the units indicated.

Course of the Reaction in Syphilitic Rabbits.—Having established the fact that the serum of normal rabbits varies greatly in the reaction to the Kahn test, it is apparent that any changes resulting from inoculation with syphilis should preferably be contrasted with the tests previous to inoculation. Ten animals tested previous to inoculation have been followed in this manner. The tests were repeated at intervals of approximately 10 days. The results are shown in table 2. This table brings out the following points.

TABLE 2
COURSE OF THE KAHN REACTION WITH SERUMS OF 10 RABBITS INOCULATED WITH SYPHILIS

Reactions (++++) in Terms of Quantitative Units														
Number of Rabbit	Tests Prior to Inocula- tion	Number of Days after Inoculation												
		10	20	30	40	50	60	70	80	90	120	150	180	
Inoculation intrates- ticular and bilateral														
1.....	++	+	4	40	40	320	320	160	160	320	160	20
2.....	0	0	±	160	320	160	80	160	160	40	40	++	0	..
3.....	0	0	0	++	320	320
4.....	+++	20	20	40	320	320	320	320	320	320	320
Inoculation on scrotum, bilateral														
5.....	..	++	++	4	80	80	80	80	320	320	320	160	40	20
6.....	0	0	20	..	80	320	320	320
7.....	20	20	20	40	20	80	80	80	40	160	80	80
8.....	0	0	0	++	40	40	20	40	4	+++	40	20	0	+++
9.....	40	40	40	20	80	80	80	40	80	80	40	20	40	..
10.....	+	++	20	20	40	40	40	20	40	4	40	+++

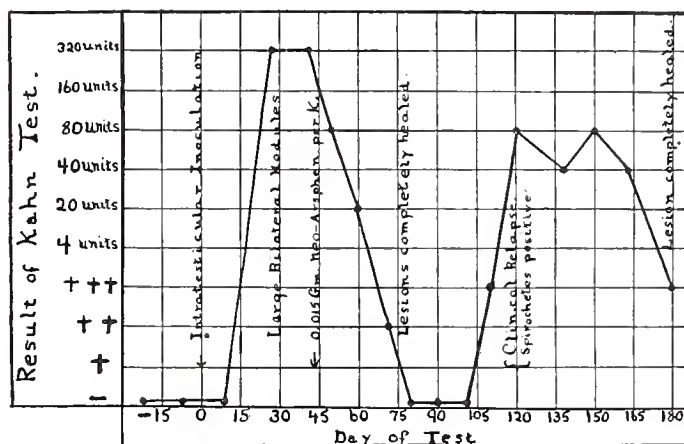
0 = no reaction and figures indicate unit strength of ++++ reaction.

Regardless of the strength of the Kahn reaction previous to inoculation, syphilitic infection brings about a marked increase in the strength of the serum reaction. In rabbit 9 (scrotal inoculation), however, the increase was only from 40 units before inoculation to 80 units after inoculation.

The strength of the reaction is influenced by the manner of inoculation. The serums of four animals inoculated intratesticularly all had reactions of 160 units or 320 units at one time. The serums of three (8, 9 and 10) of the 6 rabbits inoculated scrotally showed reactions of only 80 units or below.

The increase of the reaction to the maximum is much more prompt after intratesticular inoculation than after scrotal inoculation. After intratesticular inoculation the maximum was reached after 30 to 40 days, while after scrotal inoculation the maximum was delayed sometimes until 60 to 70 days.

After reaching a maximum, the reaction gradually becomes weaker as the infection becomes older, with a tendency for the reaction to return to negative or to the level existing prior to inoculation. The animals were, however, not followed over a long enough period to be absolutely certain of this point.



Course of the Kahn reaction in a rabbit inoculated with syphilis and then given a subcurative dose of neoarsphenamine.

It may be mentioned that, in general, the initial increase in the strength of the reaction appeared at the time of the first demonstrable lesion. Also, the period of maximum reaction corresponded with the period of maximum size of the lesions. The decrease in the strength of the reaction was accompanied by the subsidence of the lesions through spontaneous healing.

Unfortunately, the serums were not tested in dilutions higher than 1:80. It is probable that some of the rabbits had reactions greater than 320 units.

Effect of Treatment.—The effect of treatment has been followed in one animal (rabbit 3). This rabbit received 15 mg. neoarsphenamine intravenously. This dose was not curative, and the animal later showed

a relapsing lesion. This circumstance renders the experiment particularly instructive. The course of the serum reactions in this rabbit is shown in the chart. Two negative reactions were recorded before inoculation. The neoarsphenamine was injected after the development of large testicular nodules and when the serum reaction was 320 units. After the injection the lesions healed completely. The reaction gradually fell to negative, remained negative for 3 tests, and then became positive again. Examination of the rabbit showed that this was accompanied by a clinical relapse. There was a small nodule in the left testicle, in which spirochetes were found. This nodule was, however, not nearly so large as the original lesion. It persisted for a few weeks, and then disappeared. The disappearance was accompanied by a decrease in the strength of the reaction.

COMMENT

Although some authors, notably Wakerlin and Carroll,² and Kemp, Chesney, and Poole³ have found negative Wassermann tests fairly consistently in normal rabbits, the experience of many workers has been that when rabbits are tested with a Wassermann technic being used in the diagnosis of syphilis in human beings, the serums of a high proportion of the animals react positively. Generalizations in this field are difficult, however, since technical methods have varied so extensively.

One of the most recent and most elaborate studies of the serology of normal animals is that of Mackie and Watson.⁴ These workers find that the serums of a vast majority of rabbits and of other animals tested by them with the Wassermann and the Sachs-Georgi tests react positively. The difference between the positive reactions of normal animals and those of syphilitic human beings is, in their opinion, solely a quantitative difference. Further, they advance experimental evidence which leads them to believe that positive serum reactions in human syphilis are due to the augmentation of substances existing normally in serum in small amounts. The experiments here reported relating to the Kahn test with serums of normal and in syphilitic rabbits are in thorough accord with this conception of Mackie and Watson. This same point of view is also tacitly implied by Craig,⁵ Bruck⁶ and other authors who do not stress the diagnostic significance of weakly positive

² Arch. Dermatol. and Syph., 1925, 12, p. 670.

³ Bull. Johns Hopkins Hosp., 1926, 39, p. 132.

⁴ J. Hyg., 1926, 25, p. 175.

⁵ The Wassermann Reaction, 1922.

⁶ Serodiagnose der Syphilis, 1924.

reactions in human serums. In other words, the change from a normal condition to a syphilitic condition is a continuous function, and here, as in other quantitative methods, it is dangerous to name any one result and state that all results in excess of this amount denote disease. Also in accord with Mackie and Watson, I have found no constant difference in the thermolability of positive normal serum and of positive syphilitic serum.

It is evident that the Kahn test could have been modified by adding more salt to the antigen, or by other methods, so that the serums from all 29 normal animals would have reacted negatively. Due to the quantitative differences the serums of most of the syphilitic animals would still have reacted positively. But the same result can be attained arithmetically. One has merely to subtract 40 units from all the results tabulated, and the result is: all normal rabbits—"negative," and 8 out of 10 syphilitic rabbits—"positive." (The two "negative" syphilitic rabbits were inoculated scrotally.) Such a process, however, is not desirable since it conceals the essential quantitative nature of the method. It would be just as logical for biological chemists to subtract 100 mg. (per 100 cc.) from all blood sugar determinations, or for them to attempt to devise methods which fail to detect the first 100 mg.

SUMMARY

Based on a comparison of 400 presumably normal human serums and 29 presumably normal rabbit serums, a much higher incidence of positive Kahn reactions was obtained with the rabbit serums: 99.8% of the human serums, and only 36% of the rabbit serums were negative.

The Kahn reaction with serums from rabbits increases in strength following their inoculation with syphilis. The strength of the reaction is usually in direct proportion to the clinical severity of the lesion.

The increase in the strength of the reaction is more marked and more prompt after intratesticular inoculation than after scrotal inoculation. With a decrease in the size of lesions the strength of the reaction decreases.

With reservations due to the limited number of animals studied, the Kahn reaction can be made specific for syphilis in rabbits by subtracting 40 quantitative units from the results.

In the one instance observed, the Kahn reaction became negative following treatment with neoarsphenamine. A clinical relapse was accompanied by a return of the reaction.

GAS PRODUCTION BY BACTERIAL SYMBIOSIS
WITH SPECIAL REFERENCE TO THE INFLUENCE OF NITROGENOUS
SUBSTANCES

MITSUTERU ISHIKAWA

*From the Department of Bacteriology and Public Health, Washington University School of
Medicine, St. Louis, Mo.*

The study of associative actions of bacteria provides a very important as well as interesting aspect of bacteriology, since in nature various kinds of bacteria exist together under ordinary conditions. Nevertheless, not many investigators have studied the subject. Works by Nenki,¹ Burri and Stutzer,² Marshall,³ Kendall,⁴ Smith and Smith,⁵ Sherman and Shaw,⁶ Knorr,⁷ Sears and Putnam,⁸ Speakman and Philips,⁹ Kammerer,¹⁰ Castellani,¹¹ Holman and Meekison,¹² and others have contributed to our present knowledge concerning associative actions of bacteria. Further investigations, however, are required in this line to elucidate the underlying mechanism and to clarify the biologic significance of many of the phenomena. In direct connection with this paper is Kendall's description,⁴ which states that the colon bacillus forms a large amount of gas when inoculated into milk together with a bacillus of the proteolytic group, while it does not produce a noticeable amount of gas when cultivated singly in the medium. This work has been carried out to throw light on the mechanism of the symbiotic phenomenon described by Kendall and to investigate further the influences of several nitrogen-containing substances on the process of gas production.

SYMBIOSIS IN MILK

Symbiosis in Plain Milk.—So far as the available literature shows, Kendall did not try symbiotic cultures of several organisms in milk, and various combinations of ordinary microbes in milk medium have there-

Received for publication, May 21, 1927.

¹ Centralbl. f. Bakteriöl., 1. O., 1892, 11, p. 225.

² Ibid., 1894, 16, p. 815.

³ Michigan State Agricult. Coll. Spec. Bull., 1904.

⁴ Boston M. & S. J., 1910, 163, p. 322.

⁵ J. Gen. Physiol., 1920, 3, p. 21.

⁶ Ibid., 1920, 3, p. 657.

⁷ Centralbl. f. Bakteriöl., 1. O., 1922, 87, p. 536.

⁸ J. Infect. Dis., 1923, 32, p. 270.

⁹ J. Bact., 1924, 9, p. 183.

¹⁰ Klin. Wehnschr., 1924, 3, p. 723.

¹¹ Brit. M. J., 1925, 2, p. 734.

¹² J. Infect. Dis., 1926, 39, p. 145.

fore been studied. Milk is a complex medium. The organic matter of cow's milk, which was used in this work, consists chiefly of protein, carbohydrate and lipin. The protein and the carbohydrate are of special importance in connection with this work. The milk proteins are casein, lactalbumin, lactoglobulin and an alcohol soluble protein. The immunologic differentiation of these four proteins was reported by Wells and Osborne.¹³ Casein is the most abundant (3 to 4%) and characteristic protein of milk. When hydrolyzed, casein gives off many amino acids, i. e., glycocholic acid, alanine, valine, leucine, proline, phenylalanine, aspartic acid, glutamic acid, serine, cystine, tyrosine, arginine, histidine, lysine and tryptophane. The principal carbohydrate is lactose, 3 to 5%. Besides lactose, milk contains about 0.1% of a glucose-like substance. Experiments were carried out as follows:

About 15 cc. of milk were put into a Smith fermentation tube, and the tube was autoclaved. A few drops of the saturated alcoholic solution of bromocresol purple were added as an indicator to milk before tubing.

Two kinds of bacteria were inoculated simultaneously by carrying one loopful of each organism from a 0.1% glucose agar slant culture to a Smith tube. The inoculated tubes were kept in the incubator at 37 C. usually for 48 hours but sometimes for more than 72 hours. The bacteria used were *B. coli* (3 strains), *B. communior* (3 strains), *B. aerogenes*, *B. proteus*, *B. mesentericus* (3 strains), *B. pyocyaneus* and *Vibrio cholerae*. These bacteria may be divided into two groups through their ability to liquefy gelatin; the first three belong to the saccharolytic group and the rest to the proteolytic group. Various combinations were tried with an organism of the saccharolytic group and a bacterium of the proteolytic group. Gas was produced in plain milk by the following combination of organisms.

- B. coli* (1) and *B. mesentericus* (1)
- B. coli* (2) and *B. pyocyaneus*
- B. coli* (3) and *Staphylococcus*
- B. communior* (1) and *B. mesentericus* (2)
- B. communior* (2) and *B. proteus*
- B. communior* (3) and *Vibrio cholerae*
- B. aerogenes* and *B. mesentericus* (3)

Besides those shown, the following combinations were tried: *B. pyocyaneus* with other members of the saccharolytic group; and *B. coli* (1) with other members of the proteolytic group.

All of these symbiotic cultures formed acid and gas. Single cultures, one organism being inoculated into a tube, of all the bacteria employed were made as controls. Control tubes of some of the saccharolytic group demonstrated small amounts of gas, but the amount produced by the symbiosis was far greater. Milk seems to allow the saccharolytic bacteria to grow and to carry their fermentation to the production of acids but not to the vigorous formation of gas.

This series of experiments appears to confirm the observation that a saccharolytic and a proteolytic organism acting symbiotically produce gas vigorously.

¹³ J. Infect. Dis., 1921, 29, p. 200.

To clarify the importance of the proteolytic bacteria in the combinations mentioned, the following associative cultures of bacteria were tried.

B. coli with *Micrococcus ovalis*:—Gas production was not accelerated by this combination. *Micrococcus ovalis* is not proteolytic; it produces acid from the lactose of milk.

B. mucosus capsulatus with *Micrococcus ovalis*:—By this combination no gas was formed in the milk; in the usual lactose broth medium they produced acid and gas. *B. mucosus capsulatus* itself could not attack lactose.

B. mesentericus with *B. pyocyaneus*:—No gas formation was observed in this mixed culture.

The results show that neither the combinations of saccharolytic bacteria nor the mixed cultures of proteolytic bacteria produce noticeable amounts of gas in the milk. It is, therefore, clear that the proteolytic bacteria play a specific rôle for the production of gas by the saccharolytic, gas-forming bacteria.

Symbiosis in Milk with Glucose.—In the light of the first experiment it would seem that the addition of a proteolytic organism to the glucose milk medium should benefit the gas formation by a saccharolytic bacterium. The following experiment was therefore tried.

One cc. of 20% dextrose solution was added to each tube. Other cultural proceedings followed the previous experiment. The following combinations produced gas in milk with dextrose.

Morgan bacillus	and <i>B. pyocyaneus</i>	<i>B. enteritidis</i>	and <i>B. pyocyaneus</i>
	and <i>Staphylococcus</i> (1)		and <i>Staphylococcus</i> (2)
	and <i>B. mesentericus</i> (1)		and <i>Vibro cholerae</i>
<i>B. paratyphosus</i> A	and <i>B. pyocyaneus</i>	<i>B. suipestifer</i>	and <i>B. pyocyaneus</i>
	and <i>Staphylococcus</i> (1)		and <i>Staphylococcus</i> (2)
	and <i>B. mesentericus</i> (2)		and <i>mesentericus</i> (3)
<i>B. paratyphosus</i> B	and <i>B. pyocyaneus</i>	5 control tubes	produce acid
	and <i>Staphylococcus</i> (2)		
	and <i>B. mesentericus</i> (3)		

Single cultures kept as controls of bacteria of the saccharolytic group, i. e., Morgan bacillus, *B. paratyphosus* A, *B. paratyphosus* B, *B. enteritidis* and *B. suipestifer* showed slight or no production of gas.

The results show that the bacteria which can produce gas from glucose in the broth can not form noticeable amounts of gas in glucose milk medium, unless they are cultured together with proteolytic bacteria. Here again the milk medium failed to furnish suitable conditions for the production of gas from glucose by single cultures of aerogenic, nonproteolytic bacteria.

Other combinations were also tried: Morgan bacillus with other members of the proteolytic group; *B. mesentericus* (1) with other members of the saccharolytic group; Morgan bacillus with *Micrococcus ovalis*, and *B. mesentericus* (1) with *Staphylococcus* (1). In the first two

instances, all of the symbiotic cultures produced acid and gas, but in the last two cases, acid but no gas was produced. These findings also provide evidence justifying the observation that the proteolytic bacteria play an important and specific rôle in accelerating the formation of gas by saccharolytic bacteria in milk. It would seem that some factors necessary for the production of gas are lacking in the milk cultures of single, saccharolytic bacteria, and the proteolytic bacteria furnish such factors.

Effect of Interval and Viability.—Having shown experimentally that milk alone is not a suitable medium for gas production and that a combination of a saccharolytic and a proteolytic bacteria produces a large amount of gas in milk, two questions arise; first, what is the effect of an interval between two inoculations if a saccharolytic bacterium and a proteolytic organism are not inoculated simultaneously; secondly, is it necessary to keep both organisms alive to get a vigorous gas production? To answer the questions some experiments were tried with plain milk, using the combination of *B. coli* and *B. pyocyaneus*. The salient points of the result are: first, when *B. pyocyaneus* had been cultured for 24 hours before *B. coli* was inoculated the gas production occurred more intensively than when they were inoculated at the same time, but when *B. coli* had been inoculated previously *B. pyocyaneus* did not show any effect on the formation of gas; secondly, it is not necessary to keep *B. pyocyaneus* alive in association with *B. coli*, that is, the milk tube where *B. pyocyaneus* had been cultivated for 24 to 48 hours and which had been heated to kill the organism just before *B. coli* was inoculated gave a noticeable amount of gas.

<i>B. coli</i> and <i>B. pyocyaneus</i> inoculated simultaneously.....	gas
<i>B. pyocyaneus</i> inoculated previously.....	gas (larger amount)
<i>B. pyocyaneus</i> cultured and killed.....	gas (larger amount)

These findings indicate that the substances produced by the metabolic activity of a proteolytic bacterium are important in accelerating the gas production by a saccharolytic organism.

EXPERIMENT WITH CASEIN

Bacterial Action On Casein.—The formation of gas by bacterial activity in milk seems from these experiments to be dependent primarily on a change in the protein constituents of the medium. Therefore, experiments were tried to show how the bacteria used in this work act on casein, the most important protein of milk. Using milk agar plates,

Hastings¹⁴ found that *B. coli* and Friedländer's bacillus were unable to attack casein. Rettger and others¹⁵ conclude that even the gelatin-liquefying bacteria cannot act on pure proteins.

The medium was prepared as follows:—5.0 gm. of sodium chloride, 1.0 gm. of dipotassium hydrogen phosphate, 0.5 gm. of dextrose, and 0.1 gm. of bacto peptone were dissolved in 1000 cc. of tap water; the reaction was adjusted to P_H 7.2 after boiling; 4 cc. of this solution were poured into each of several test tubes; the tubes were plugged with cotton and autoclaved. To each tube was added 2 cc. of 1% neutral casein solution which had been filtered through a Berkefeld filter.

The bacteria were inoculated from agar slant cultures, one loopful of an organism being carried to each tube. After incubation for one week at 37°C. the cultures were tested by the method which had been proved by Frazier¹⁶ to be satisfactory for the detection of the change of gelatin by bacteria. The principle of this method is a modification of the formol titration for protein hydrolysis. The results with bacteria in 1% neutral casein medium were as follows.

NO INCREASE IN AMINO NITROGEN	INCREASE IN AMINO NITROGEN
<i>B. coli</i> (strains 1, 2 and 3)	<i>Staphylococcus</i> (strains 1 and 2)
<i>B. communior</i> (strains 1, 2 and 3)	<i>B. pyocyaneus</i>
<i>B. aerogenes</i> (strains 1 and 2)	<i>B. proteus</i>
<i>B. mucosus</i>	<i>B. mesentericus</i>
<i>Morgan bacillus</i>	<i>Vibrio cholerae</i>
<i>B. paratyphosus A</i>	
<i>B. paratyphosus B</i>	
<i>B. enteritidis</i>	
<i>B. suispestifer</i>	
<i>B. foetidus</i>	
<i>Micrococcus ovalis</i>	

So far as this test proves, of the bacteria used in this work, none of the saccharolytic group can attack casein, while all of the proteolytic group are able to break down casein. The results with serum albumin and egg white were the same as with casein.

This test alone cannot be regarded as of more than qualitative chemical accuracy for the detection of changes of proteins. Moreover, theoretically, the fact that a certain organism fails to attack casein does not prove of course that it cannot break down other milk proteins, such as albumin, globulin and alcohol soluble protein. But the results of the extensive investigation by Kendall, Day and Walker¹⁷ on the nitrogen metabolism of various bacteria in milk proved that *B. alkaligenes*, *B. typhosus*, *B. paratyphosus A*, *B. paratyphosus B*, *B. coli* and *B. cloacae* leave milk proteins practically unattacked even after a prolonged incubation, and that *B. proteus*, *B. subtilis*, *B. mesentericus*, *Vibrio cholerae*, *B. pyocyaneus* and *Staphylococcus* do very definitely attack milk proteins to produce ammonia. So that it may safely be concluded that none

¹⁴ Centralbl. f. Bakteriöl., 1, O., 1904, 12, p. 590.

¹⁵ Sperry and Rettger: J. Biol. Chem., 1915, 20, p. 445. Rettger, Berman and Sturges: J. Bact., 1916, 1, p. 15.

¹⁶ J. Infect. Dis., 1926, 39, p. 302.

¹⁷ J. Am. Chem. Soc., 1914, 36, p. 1937.

of the bacteria of the saccharolytic group studied here is able to attack milk proteins to a significant degree and all members of the proteolytic group herein considered do cleave at least casein.

EXPERIMENTS WITH MILK CONTAINING ADDITIONAL NITROGENOUS SUBSTANCES

So far, the following points have been shown:

First, the saccharolytic bacteria, when cultured singly, do not produce noticeable amounts of gas in plain milk or in glucose milk. Second, the saccharolytic bacteria produce gas intensively in the milk mediums, if they are cultured in symbiosis with the proteolytic bacteria. Third, substances produced from some constituents of milk by the metabolic activity of the proteolytic organisms benefit the production of gas from glucose or lactose by the saccharolytic bacilli. Fourth, the saccharolytic bacilli do not attack the milk proteins to a noticeable extent, while the proteolytic bacteria are able to break down definitely the proteins, at least casein, of milk.

Thus, it may logically be considered that in symbiosis in milk the proteolytic bacteria attack the proteins, at least the casein, and the digestion products of the proteins favour the formation of gas by the saccharolytic bacilli. It was, therefore, intended in the following experiments to investigate influences of digestion products of proteins and of other simple nitrogenous compounds upon the formation of gas by single cultures of the saccharolytic bacilli in milk.

Milk was used in two ways: in the Smith fermentation tube and in the agar tube. Thirteen cc. of milk was put into a Smith tube. As an alternative medium one part of milk was mixed with one part of neutralized 2.0% aqueous solution of agar; 7 cc. of this mixture was poured into an ordinary test tube. Bromcresol purple was used as the indicator. Various solutions were added, 2 cc. to a Smith tube and 1 cc. to an agar tube, after being neutralized. Inoculation and incubation were performed as before.

Products of Tryptic and Peptic Digestion of Proteins.—It is established that trypsin in the alkaline solution (about P_H 8.0) digests proteins to amino acids, and that pepsin works in the acid solution (about P_H 2.0) to produce proteoses and peptones from proteins, amino acids also being formed after a prolonged digestion.

Milk: Two lots of milk were digested by trypsin and pepsin respectively under proper conditions, for 18 hours at 37 C. The products were added to tubes, and the tubes were autoclaved. The results are tabulated.

	Plain	Trypsin digestion	Pepsin digestion
B. coli (strains 1, 2 and 3), B. communior (strains 1, 2 and 3), and B. aerogenes.....	Acid	Gas	Gas
B. foetidus	Acid	Acid	Acid
B. alkaligenes	No acid or gas		

B. foetidus produces only acid in the lactose broth; *B. alkaligenes* is unable to ferment any sugar. These bacteria were included as controls. The amount of gas produced in the medium containing the trypsin-digested products was distinctly greater than that in the medium containing pepsin-digested products.

Serum Albumin (Horse); Egg White (Hen); and Casein:—1% solutions of these substances were treated in the same manner as milk, that is, they were digested by trypsin and pepsin, and the products were added to milk. The results were practically the same as with digestion products of milk.

Briefly speaking, the digestion products of milk, serum albumin, egg white and casein by trypsin or pepsin accelerate the production of gas by the saccharolytic bacteria in milk, but they do not show any noticeable effect on the activity of non-gas-producing bacteria, such as *B. foetidus* and *B. alkaligenes*.

Peptone.—The fact that the gas-producing bacteria produce large amounts of gas in the peptone water medium and the results with digested proteins led to this experiment. A 1% solution of bacto-peptone was used. Almost the same results, which are shown in table 1, were obtained as with the above mentioned digestion products, but peptone was more effective in giving rise to the formation of gas.

TABLE 1
EFFECTS OF PEPTONE AND AMINO ACIDS IN THE MEDIUMS

	Peptone Gas (++)	Alanine Gas (+)	Glutamic Acid Gas (+)	Urea Gas, (small amount) Acid
<i>B. coli</i> , <i>B. communior</i> and <i>B. aerogenes</i> ,.				
<i>B. foetidus</i>	Acid	Acid	Acid	
<i>B. alkaligenes</i>		No acid and no gas		

Amino Acids and Urea.—Since peptone added to milk allowed vigorous gas production by the saccharolytic bacteria, simpler nitrogenous compounds were tested. Solutions (5 per cent) of alanine, glutamic acid, asparagine, and urea were prepared with distilled water, and neutralized. Asparagine was used in a hot solution, because of its low solubility. The solutions, except the urea solution which was sterilized by filtration and added to autoclaved tubes, were added to milk tubes before sterilization.

Some of the results are shown in table 1. *B. coli*, *B. communior*, and *B. aerogenes* produced noticeable amounts of gas in the milk containing alanine, glutamic acid, asparagine or urea, although urea was weaker in the effect than the other substances, and that peptone showed far greater effect than any of them. These substances did not affect *B. foetidus* and *B. alkaligenes*.

Ammonium Salts.—Recently Zeller¹⁸ published his study of the influences of ammonium salts on fermentation by yeasts. He found that several ammonium salts, such as acetate, bromide, carbonate, chloride, formate, iodide, molybdate, nitrate, oxalate, phosphate and sulphate, accelerate, but ammonium benzoate retards the yeast fermentation.

In this experiment the following ammonium salts were used: the bromide, carbonate, chloride, phosphate and sulphate. Solutions (5%) of these substances were prepared, which were added to milk tubes before sterilization. The results with the different bacteria may be summarized as follows:

B. coli, B. communior, and B. aerogenes.....	gas	} in the chloride, bromide, phosphate and sulphate, ammonium salts
B. foetidus.....	acid	
B. alkaligenes.....	no acid or gas	

The ammonium carbonate gave inconstant results.

Combinations of Nitrogen Compounds.—Since peptone added to milk stimulated the formation of gas noticeably more than amino acids and ammonium salts were quite favorable for gas formation, combinations of amino acids and ammonium salts were tried: alanine with glutamic acid, glutamic acid with urea, glutamic acid with ammonium chloride, and ammonium phosphate with asparagine, but none of these combinations proved to be superior to any single one of them.

Milk with Glucose.—In experiments similar to those just described, milk containing 1% glucose was used with the Morgan bacillus, B. paratyphosus A, B. paratyphosus B, B. enteritidis and B. suispestifer. Results corresponded to those of the previous experiments, and the details are omitted.

EXPERIMENTS WITH SYNTHETIC MEDIUMS

The importance of amino acids and of ammonium compounds for the production of gas by the saccharolytic bacteria in milk indicates how necessary is the addition of the simpler nitrogenous substances for the vigorous production of gas by these bacteria, but not for their growth and for their production of acids. Milk, however, is far more complicated in its composition than other ordinary culture mediums, so to bring the points into a simpler and clearer field experiments with synthetic mediums were performed.

Gordon¹⁹ found that when glucose is present in a medium as a source of carbon, B. coli, B. paratyphosus, B. pyocyaneus and B. proteus satisfy their nitrogen requirement for growth with either ammonium salts, amino acids

¹⁸ Biochem. Ztschr., 1926, 175, p. 135.

¹⁹ J. Royal Army Med. Corps, 1919, 28, p. 3071.

(glycocoll, alanine and aspartic acid) or asparagine, but *B. typhosus*, Friedländer's bacillus, *B. diphtheriae*, *B. pseudodiphtheriae* and several species of staphylococcus and streptococcus fail to do so, and that *Vibrio cholerae* and *B. dysenteriae* (Flexner) can utilize certain of the amino acids but not the ammonium salts. Koser and Rettger²⁰ proved that amino acids, such as histidine, phenylalanine, lysine, aspartic acid and glycocoll, are able to furnish nitrogen to bacteria, and that combinations of amino acids and other nitrogen containing compounds have little value over any of the single amino acids. But Gordon, Koser and Rettger apparently investigated only from the standpoint of the bacterial nutrition or growth.

The base of the medium was composed of sodium chloride, 5.0 gm.; magnesium sulphate, 0.2 gm.; calcium chloride, 0.01 gm.; monobasic acid potassium phosphate, 1.0 gm.; distilled water, 1000 cc. This base was used both in fluid and solid mediums. In the latter, agar was added to make the concentration 1%. Thirteen cc. of the fluid were poured into a Smith tube and 7 cc. of the agar into a test tube. The reaction was adjusted to P_H 7.2 before tubing, and a few drops of a saturated alcoholic solution of bromcresol purple were added as the indicator. Sterile sugar solutions were added to the sterilized tubes to make the final sugar concentration approximately 1%.

The following substances were tried as the source of nitrogen for growth and fermentative activity of microbes: digested milk, digested serum, digested casein, peptone, alanine, asparagine, glutamic acid, urea, ammonium salts (the acetate, borate, bromide, carbonate, chloride, iodide, lactate, molybdate, nitrate, oxalate, phosphate, succinate, sulphate, tartrate, benzoate, salicylate), anilin, codein sulphate, caffeine, hexamethylamine, phenacetin, potassium cyanide, saccharin, and sulphanilic acid. Among these substances, the first eight were used in the same way as in previous experiment: the second fourteen at the concentration of 0.3% and the last ten at lower concentrations, since many of the last group are toxic or sparingly soluble.

The comparison between two ways of inoculation was tried with the colon bacillus; the one with a loopful of the broth culture, and the other with a loopful of the heavy suspension in the normal salt solution of the bacteria grown on agar slants. No difference was observed between the two methods, and it was decided to inoculate one loopful of the broth culture of an organism into a tube. Inoculated tubes were kept in the incubator at 37 C., usually for 48 hours, but sometimes for one week to ascertain results. Smith tubes and agar tubes were used for the same experiment, when conditions permitted, to check up results.

With Glucose.—Some of the results are shown in table 2. Besides *B. coli*, *B. communior*, *B. aerogenes*, Morgan bacillus and *B. pyogenes-foetidus*, which are listed in the table, *B. paratyphosus* A, *B. paratyphosus* B, *B. enteritidis*, *B. suipestifer* and *B. proteus* were also tried. All of them, except *B. foetidus* which is unable to form gas in any medium, showed practically the same results.

Tubes containing digested proteins (milk, serum, or casein), urea, peptone, alanine, asparagine, glutamic acid, or ammonium salts (acetate, borate, bromide, carbonate, chloride, iodide, lactate, molybdate, oxalate, phosphate, succinate, sulphate or tartrate) showed growth of bacteria and production of acid and gas, although the tubes of urea, ammonium acetate and ammonium carbonate gave

²⁰ J. Infect. Dis., 1919, 24, p. 301.

rather inconstant results. In the tubes of ammonium or potassium nitrate, there appeared a heavy growth of bacteria and a slight indication of acidity, but gas was not produced.

Ammonium benzoate, ammonium salicylate, anilin, caffeine hexamethylamine, phenacetin, potassium cyanide, saccharin and sulphanilic acid were tried at various concentrations, but none of them allowed bacteria to grow. Some of the colon bacilli scantily grew and produced small amounts of gas in the 0.3% codein sulfate medium, but not enough experiments were carried out to draw a definite conclusion.

TABLE 2
SYNTHETIC MEDIUMS CONTAINING GLUCOSE AND NITROGENOUS SUBSTANCES

Nitrogenous Substances	Morgan Bacillus	B. Aerogenes	B. Foetidus
Digested proteins (milk, serum or casein), alanine, asparagine, glutamic acid or am- monium salts (borate, chloride, lactate, molybdate, oxalate, succinate, tartrate)....	Gas	Gas	Acid
Peptone	Gas (larger amount)	Gas (larger amount)	Acid
Urea	Gas (small amount)	Gas	Acid
Potassium nitrate	Acid (slight)	Acid (slight)	Acid (slight)

Lactose with *B. coli*, *B. communior*, *B. aerogenes* and *B. acidilactici*; sucrose with *B. communior*, *B. aerogenes*, *B. mucosus* and *B. proteus*; maltose with *B. coli*, *B. aerogenes* and *B. enteritidis*, and mannitol with *B. paratyphosus* A, *B. paratyphosus* B, *B. coli* and *B. aerogenes* were tested, with practically the same results as with glucose. Therefore, the details are not described.

Combinations of Nitrogenous Substances.—Using *B. coli* and Morgan bacillus, combinations of simple nitrogenous compounds were tried in several ways: alanine with ammonium chloride, urea with ammonium chloride, ammonium bromide with ammonium chloride, and potassium nitrate with peptone, glutamic acid, asparagine, ammonium chloride, lactate, or succinate.

In the first three cases, the combinations did not exhibit any noticeable advantage in either gas production or bacterial growth. Interesting results were obtained from the fourth case. So far as the production of gas was concerned, potassium nitrate worked antagonistically against other nitrogenous compounds. When 1 cc. of the 5% solution of potassium nitrate and 1 cc. of the 5% solution of any of the amino acids and ammonium salts were added to a Smith tube, gas formation did not take place, in spite of heavy growth of bacteria.

Concentrations of Nitrogenous Substances.—The effects of different concentrations of alanine, glutamic acid, ammonium chloride and

ammonium phosphate upon the production of gas by the colon bacillus were next studied.

The synthetic medium contained 1% glucose, and the nitrogenous substances listed above were used at the percentages of 0.1, 0.5, 1.0, 2.0 and 3.0. Only Smith tubes were used in this experiment. The results are tabulated, with (+), +, and ++ indicating increasing amounts of gas.

	% — 0.1	0.5 and 1.0	2.0	3.0
Alanine	(+)	++	+	(+)
Glutamic acid	(+)	++	++	+
Ammonium chloride	(+)	++	+	(+)
Ammonium phosphate	(+)	++	+	(+)

These results indicate that the proper concentration of these nitrogenous substances lies between 0.5% and 1.0%.

Symbiosis.—Sears and Putnam,⁸ Castellani¹¹ and Holman and Meekison¹² carried out experiments using various combinations of gas-forming bacteria with acid producing organisms, and found that in the peptone water or the broth containing a certain carbohydrate, acid and gas are produced when the mediums are inoculated with two kinds of bacteria, the one of which is able to produce acid and gas from glucose but cannot ferment that carbohydrate, and the other is able to produce acid from that carbohydrate. An experiment was performed to determine if the same phenomenon occurs also in the synthetic mediums.

To the synthetic base containing 1% lactose, alanine, glutamic acid or ammonium chloride was added as the source of nitrogen to make the concentration of 0.5%. Combinations of Morgan bacillus with *Micrococcus ovalis* and with *B. foetidus* were tried. The gas-forming organism and the acid-producing organism were inoculated simultaneously. After the incubation of from 24 to 48 hours, all of the cultures showed production of acid and gas, although there was neither heavy growth nor large amounts of gas to be compared with those of broth cultures.

The formation of gas by bacterial symbiosis evidently takes place in the synthetic mediums containing an amino acid or an ammonium salt as well as in more complex mediums.

COMPARISON OF EFFECTS OF SALTS

Holm and Sherman²¹ showed that Cl, I, NO₃, SO₄, and PO₄ ions, when used at 0.2 molar concentration of salts, have beneficial effects upon the growth of bacteria, and Winslow and Falk²² stated that sodium chloride, when used at the concentrations from 0.0145 to 0.725 molar, favors the viability of the colon bacillus. Previous results have shown that several ammonium salts accelerate the formation of gas in milk by

²¹ J. Bact., 1921, 6, p. 511.

²² Ibid., 1923, 8, p. 215.

the saccharolytic bacteria, and that, as the sole source of nitrogen, they satisfy the requirements of such bacilli for growth and for fermentative activity. Thus, it was desired to find out if there is any difference between ammonium salts and other salts in the influence on gas production by *B. coli*, *B. communior*, and *B. aerogenes*.

Sodium Chloride and Ammonium Chloride.—The bacteria were inoculated into Smith tubes containing milk, to which sodium chloride or ammonium chloride had been added to make M/10 concentrations. Tubes of sodium chloride produced larger amounts of gas than control tubes, but less amounts than ammonium chloride tubes. When 1% peptone water containing 1% of lactose was used instead of the milk, the difference in amounts of gas was not definite, some of the sodium chloride tubes giving rise to greater amounts than ammonium chloride tubes.

Potassium Phosphate and Ammonium Phosphate.—In the second experiments, dibasic potassium phosphate and ammonium phosphate were compared. The medium consisted of 0.1% peptone, 0.5% sodium chloride, 1.0% dextrose and potassium phosphate or ammonium phosphate, M/10. Larger amounts of gas were produced in the tubes of ammonium phosphate than in the tubes of potassium phosphate, and the latter contained greater amounts than control tubes. But when peptone was used at 1.0% instead of 0.1%, the results were rather inconstant. The results are tabulated.

	Milk Gas (small amount)	0.1% Peptone	1.0% Peptone Gas
Sodium chloride			
Ammonium chloride	Gas		Gas
Potassium phosphate		Gas (small amount)	Gas
Ammonium phosphate		Gas	Gas

These results suggest that in the mediums containing insufficient amounts of simple nitrogen substances, ammonium chloride and ammonium phosphate have greater beneficial effects on the formation of gas than sodium chloride and potassium phosphate, but in the mediums which contain sufficient nitrogen substances there is almost no difference in the effects between the former and the latter. Besides, it is obvious that ammonium chloride and ammonium phosphate furnish nitrogen to bacteria for their growth and activity, as shown in the experiments with synthetic media, but sodium chloride and potassium phosphate are unable to do so.

EXPERIMENTS WITH PROTEIN MEDIUMS

Several experiments with protein mediums, such as ascitic fluid, casein, nutrose, and gelatin mediums, were also conducted.

Ascitic fluid is a transudate, the protein contents (5.0—1.0%) of which are albumin and globulin. It also contains nonprotein organic substances and other components of the blood plasma. About 0.1% glucose is ordinarily present. The quantities of those constituents vary somewhat in different lots of the fluids.

Casein contains many kinds of amino acids as stated before. Nutrose is sodium caseinate. Gelatin also contains many amino acids but it lacks cystine and tyrosine.

Procedure.—One part of ascitic fluid was added to two parts of sterile distilled water in the aseptic way. Casein, nutrose and gelatin were dissolved in the synthetic base mentioned before to make the concentration of 0.5%, and the solutions were sterilized by passing through the Berkefeld filter. All the mediums were adjusted to about P_H 7.2, and bromcresol purple solution was added to them as the indicator. Sugars were used at 1.0% concentration. In most cases Smith fermentation tubes were used, and in some cases Durham tubes. Inoculation and incubation were performed in the same way as in the experiments with synthetic mediums.

Single Culture.—The fermentation of lactose by the colon bacillus was first studied. *B. coli* communis grew heavily and produced a strong acidity and just a trace of gas in the ascitic fluid tube. But, the symbiotic culture of the colon bacillus with *B. mesentericus* produced a considerable amount of gas in the tube. In one lot of ascitic fluid, however, the colon bacillus produced a large amount of gas. That fluid was not used in the further experiments, since it was not suitable for the purpose.

The colon bacillus grew scantily and produced acid reactions in the casein, nutrose and gelatin mediums. But, when *B. pyocyaneus* or *B. mesentericus* had been cultivated in these mediums for 24 hours before the inoculation of the colon bacillus, there occurred heavy growth and vigorous production of acid and gas in the tubes of the casein, nutrose and gelatin mediums. The proteolytic organisms alone gave the alkaline reaction but no gas in these mediums, even after a long incubation. When the proteolytic organism and the colon bacillus were inoculated simultaneously the results were not constant, unless the proteolytic bacterium was inoculated very heavily.

Also, the colon bacillus alone was able to produce gas in the ascitic fluid, casein, nutrose and gelatin mediums, provided that peptone, an amino acid or an ammonium salt had been added to them, as was the case with milk thus enriched.

Associative Cultures.—Morgan bacillus in association with *Micrococcus ovalis* produces acid and gas in the lactose broth. But the combined culture of these two organisms did not produce a noticeable amount of gas in the ascitic medium containing lactose, although they grew luxuriantly and produced acids. In the casein, nutrose and gelatin mediums they did not grow demonstrably. However, they grew well and produced acid and gas in all of those mediums, to which peptone, an amino acid or an ammonium salt had been added.

Further, when *B. pyocyaneus* or *B. mesentericus* had been cultivated in such mediums before the inoculations of Morgan bacillus and *Micrococcus ovalis* they grew fairly well and produced acid and gas. The combinations of the proteolytic bacteria with the associative cultures of two kinds of saccharolytic bacteria, such as *B. coli* with *Micrococcus ovalis* and *B. paratyphosus* with *B. dysenteriae* (Flexner), in the ascitic medium containing sucrose or mannitol were also tried. The results are shown in the tabulation.

Morgan bacillus and <i>Micrococcus ovalis</i>	acid	} lactose
Morgan bacillus, <i>Micrococcus ovalis</i> and <i>B. pyocyaneus</i>	acid + gas	
<i>B. paratyphosus</i> A and <i>B. dysenteriae</i> (Flexner).....	acid	} mannitol
<i>B. paratyphosus</i> A, <i>B. dysenteriae</i> and <i>B. mesentericus</i>	acid + gas	
<i>B. coli</i> and <i>Micrococcus ovalis</i>	acid	} sucrose
<i>B. coli</i> , <i>Micrococcus ovalis</i> and <i>B. pyocyaneus</i>	acid + gas	

It should be recalled that the synergic cultures of Morgan bacillus with *Micrococcus ovalis*, of *B. paratyphosus* with *B. dysenteriae* (Flexner) and of *B. coli communis* with *Micrococcus ovalis* produce acid and gas from proper carbohydrates in peptone water and broth mediums. These combinations, however, failed to form gas in the ascitic fluid mediums. Through the assistance of the proteolytic organisms, however, they produced noticeable amounts of gas in the mediums.

Two kinds of saccharolytic bacteria must be living together, but it is not necessary to keep the proteolytic bacteria alive. Cultures of the proteolytic bacteria, which have been incubated for from 24 to 48 hours and were killed by heat, gave the same effect as the living cultures in facilitating gas formation by the symbiosis of saccharolytic bacteria, as could be expected from the work on the effect of "interval and viability."

It seems that the associative action of three kinds of bacteria—a proteolytic bacterium, an acid-producing saccharolytic bacterium and a gas-forming, saccharolytic organism—has never been described.

B. Proteus.—The third experiments were with *B. proteus*. In this case glucose was used instead of lactose.

B. proteus grew heavily and produced an acid reaction but practically no gas in the ascitic, casein, nutrose and gelatin mediums; but produced acid and gas in those mediums, when they contained peptone, an amino acid or an ammonium salt. It also produced acid and gas in tubes of those mediums, in which *B. mesentericus* or *B. pyocyaneus* had been cultivated. Moreover, when, *B. proteus* had been inoculated heavily into those mediums and the tubes had been incubated for 24 hours and then glucose was added, there was the production of acid and gas.

Additional evidence is thus provided for the importance of suitable nitrogen substances for the completion of fermentative processes. It may be assumed that the simple nitrogenous substances contained in the ascitic fluid were sufficient for the microbic growth and for the production of acids by the saccharolytic bacteria but insufficient for the formation of gas, and that the casein, nutrose and gelatin mediums did not have sufficient amounts of such substances even for the growth of the nonproteolytic bacteria. As described above, one lot of ascitic fluid was discarded for the reason that the colon bacillus produced a fairly large amount of gas in it. That lot of the fluid probably had greater amount of simple nitrogenous substances which favored the formation of gas more than the other lot.

Additional nitrogenous substances, such as peptone, amino acids and ammonium salts must have furnished the nitrogenous substances essential for the production or the activity of the enzyme, which splits an intermediate product of fermentation into gases.

The proteolytic bacteria must have decomposed proteins into simpler forms, which worked in the same way as the additional nitrogen substances. The fact that it is not necessary to keep the proteolytic bacteria alive for accelerating the production of gas by the saccharolytic bacteria indicates that the beneficial effect of those bacteria are dependent rather upon the products of proteolysis than upon their subsequent viability.

One of the results of experiments with *B. proteus*—the proteus bacillus produces acid and gas markedly if glucose is added after it has grown, while if the inoculation and the addition of the sugar were performed at the same time there occurs acid production but almost no gas formation—can be explained by the principle of the protein sparing action of the carbohydrate, which has been chiefly established by Kendall and his associates. Thus, if the proteus bacillus is inoculated into the glucose medium, the bacillus attacks the carbohydrate first and does not act catabolically on the protein substances; therefore, the simple forms of nitrogen substances have not been produced by its activity when it needs them for the completion of its fermentation. But, if the bacillus has been cultured before the addition of the sugar, the proteins of the medium have been attacked and the simple forms of nitrogen substances are abundant when the fermentation takes place; a considerable amount of gas is generated in the presence of such simple nitrogenous substances.

DECOMPOSITION OF SODIUM FORMATE

According to the investigations of Franzen and Stuppahn,²³ Franzen and Braun²⁴ and Frankland and others,²⁵ the formation of gas by bacteria from carbohydrates appears to result from the decomposition of formic acid, which is an intermediate product of fermentation, into hydrogen and carbon dioxide. Pakes and Jollyman²⁶ found that *B. coli*, *B. enteritidis*, the pneumobacillus of Friedländer, *B. lactis-aerogenes*, *B. cloacae*, *B. proteus* (*vulgaris*) and *B. prodigiosus* are able to decompose formic acid into hydrogen and carbondioxide. They used the broth medium containing meat infusion, peptone, sodium chloride and sodium formate. It was, therefore, intended to see how the gas producing bacteria act on sodium formate in different mediums.

In the following experiments *B. paratyphosus* A, *B. paratyphosus* B, Morgan bacillus, *B. enteritidis*, *B. supestifer* and *B. mucosus* were used. A solution of sodium formate (20%) was sterilized by passing it through the Berkefeld filter, and added to sterile medium to make approximately 1%.

With Broth.—In the broth prepared in the ordinary way all of the bacteria mentioned above produced fairly large amounts of gas from sodium formate after incubation of from 12 to 24 hours.

With Milk.—In the milk containing sodium formate all of the bacteria produced but traces of gas, while they produced a slight acidity but no gas in plain milk.

When they were inoculated together with an organism of the proteolytic group, such as *B. pyocyaneus* and *B. mesentericus*, into the formate milk, there appeared fairly large amounts of gas. These proteolytic bacteria did not produce gas from sodium formate.

All of the bacteria, when cultured singly, also produced gas noticeably in formate milk containing an additional nitrogen substance, i. e., peptone, an amino acid or an ammonium salt.

²³ Ztschr. f. physiol. Chem., 1912, 77, p. 129.

²⁴ Ibid., 1908, 8, p. 29.

²⁵ J. Chem. soc., 1892, 61, p. 270.

²⁶ Ibid., 1901, 79, p. 386.

The results of these experiments, tabulated below, indicate that simple nitrogenous substances are necessary to allow the bacteria to act upon sodium formate.

Each of Following Organisms	Plain Broth	Plain Milk	Milk with Additional Nitrogenous Substances	Milk with Proteo- lytic Bacteria
B. paratyphosus A, B. paratyphosus B, Morgan bacillus, B. enteritidis, B. supestifer, B. mucosus.....	Gas	No Gas	Gas	Gas

With Synthetic Medium.—The bacteria did not grow satisfactorily in the synthetic medium containing sodium formate and an amino acid or an ammonium salt, but when there was a fair growth, the production of gas occurred. Peptone, however, allowed the bacteria to grow and to produce gas from sodium formate in the medium. In some tubes ammonium formate instead of sodium formate was added to the synthetic base with the idea that ammonium formate might furnish the nitrogen on the one hand and the source of gas on the other. But the production of gas did not occur, apparently through the lack of growth.

With Protein Mediums.—Preparation of ascitic fluid, casein, nutrose and gelatin mediums was carried out in the same manner as described before, except that the sodium formate solution was used at the final concentration of 1.0% in place of sugar solutions.

In the ascitic fluid medium, the bacteria grew luxuriantly to produce only traces of gas, but these saccharolytic bacilli formed large amounts of gas when proteolytic bacteria, as *B. mesentericus* and *B. pyocyaneus*, were cultured together with them, and when peptone, an amino acid or an ammonium salt was added to the tubes. In the casein, nutrose and gelatin mediums the bacteria did not multiply noticeably. However, they grew fairly well and formed demonstrable amounts of gas in these mediums, provided that the proteolytic bacteria had been cultured in the medium or one of the simple nitrogenous substances was added.

These results indicate that the presence of the nitrogen substances, such as peptone, amino acids and ammonium salts, has the beneficial effect upon the formation of gas from sodium formate by bacteria, in other words, on the activity or the production of formiase. The assumption that the amount of amino acids is more abundant in the broth than in the milk and the ascitic fluid mediums will account for the larger amount of gas produced in the broth. The beneficial effects of the nitrogenous substances added intentionally and of the association of proteolytic bacteria with the gas forming bacilli, on the decomposition of sodium formate in milk, ascitic, casein, nutrose and gelatin mediums are explained by the same principle. These protein mediums do not contain enough simple nitrogenous substances for the activity or the production of formiase. If, therefore, one of the nitrogen substances is added or digestion products of the milk proteins by the proteolytic bacteria are present, the activity or the production of the formiase is greatly favored.

In summarizing, it may be stated that the production of gas from sodium formate by the saccharolytic bacilli, that is, the production or the activity of formiase, requires simple types of nitrogenous substances, either produced by the proteolytic bacteria from proteins of the mediums or added purposely to the mediums.

DISCUSSION

The importance of the simple types of nitrogenous substances, such as the digested products of proteins, peptone, amino acids and ammoniums salts, for the production of gas from carbohydrates and salts of formic acid by bacteria is shown in the results of this series of experiments. It is, however, impossible to formulate the precise relations existing between these substances and fermentative processes. This must await the isolation of fermentative enzymes themselves. Moreover, the anabolic and the catabolic phases of bacterial metabolism cannot be quantitatively differentiated by existing methods, which makes it difficult to study certain aspects of the problem which has been taken up in this work. Nevertheless, the inseparable relationship between the production of gas and the character of the essential nitrogenous substances has been illustrated in various experiments.

Thus, in the study of associative actions of bacteria, it was shown that a bacterium of the saccharolytic group produces a large amount of gas in the milk, ascitic fluid, casein, nutrose and gelatin mediums, if it is cultured in symbiosis with an organism of the proteolytic group, and also that one proteolytic organism and two kinds of saccharolytic bacteria—one, acid-producing, and the other, gas-forming—can produce gas in such protein mediums. The mechanism involved in this phenomenon may be explained simply by stating that the proteolytic organism paves the way for the production of gas by a saccharolytic bacterium or by the symbiosis of two kinds of saccharolytic bacteria. The proteolytic bacterium attacks the proteins of mediums to produce simple forms of nitrogenous substances which appear to be indispensable to the production of gas.

It was also found that the digestion products of proteins, peptone, amino acids and ammonium salts accelerate the formation of gas from carbohydrates, or from sodium formate, by single cultures of aerogenic bacilli or by synergic cultures of two species of saccharolytic bacteria in the complex protein mediums, such as milk, ascitic fluid, casein, nutrose and gelatin. It is probable that the beneficial effect of the digestion products and of peptone is chiefly due to amino acids contained in them,

since the end products of tryptic digestion of proteins are simpler peptides and amino acids; also, it should be recalled that the commercial peptones ordinarily contain free amino acids.

Just how such simple forms of nitrogenous substances exert a favorable influence on the formation of gas is not precisely answered, but there are some evidences which seem to justify the belief that these substances accelerate particularly the production or the activity of the specific enzyme, formiase. The steps are: first, the colon-aerogenes group of bacteria ferment lactose into acids in milk and ascitic fluid mediums, but they do not generate gas noticeably from sodium formate in such protein mediums, second, carbohydrates and sodium formate are decomposed into gases by them in milk, ascitic fluid, casein, nutrose and gelatin mediums, if simple nitrogenous substances are added intentionally to the mediums, or if the proteolytic bacteria are cultivated in association with them, and third, benzoates, which inhibit the decomposition of sodium formate do not allow gas-producing bacteria to carry the fermentation of carbohydrates to gas production; they stop the process at the production of acids. Herter²⁷ reported that sodium benzoate has the property to inhibit the production of gas from glucose by the colon bacillus. An experimental study of the inhibitory actions of benzoates and other chemicals upon the production of gas by bacteria will be published elsewhere. The inhibitory action of the benzoates on the production of gas as mentioned above indicates that the production of acid and the formation of gas by aerogenic bacteria is carried on by different enzymes. According to Franzen and others, the enzyme responsible for the production of gas from carbohydrates is formiase.

SUMMARY

The nonproteolytic, gas-forming bacteria alone cannot produce noticeable amounts of gas from carbohydrates and salts of formic acid in mediums containing only complex nitrogenous substances, as milk, ascitic fluid, casein, nutrose and gelatin, but gas is generated intensively by these bacteria in such mediums if they are grown in association with the proteolytic bacteria, and demonstrable amounts of gas are formed if simple forms of nitrogenous substances—the digestion products of proteins, peptone, amino acids and ammonium salts—are added to such mediums.

²⁷ J. Biol. Chem., 1909, 7, p. 59.

The formation of gas from carbohydrates by the associative cultures of two kinds of saccharolytic bacteria, an acid-producing organism and a gas-forming bacterium, does not take place markedly in ascitic fluid, casein, nutrose and gelatin mediums, except under the following conditions: When a proteolytic organism is cultivated in association with the two saccharolytic bacteria; or when simple nitrogenous substances—peptone, amino acids or ammonium salts—are added to the mediums.

The rôle played by the proteolytic bacteria in the above mentioned cases is that they pave the way for the fermentative activity of the saccharolytic bacteria by breaking down the proteins of the mediums into simple forms of nitrogenous substances which apparently are essential to the formation of gas.

It is probable that such simple nitrogenous substances accelerate the activity or the production of the specific enzyme, formiase, which is thought to be responsible for the production of gas.

Many of the ammonium salts, as the sole source of nitrogen, satisfy the bacterial requirements for growth and for fermentative activity, but ammonium benzoate and ammonium salicylate are unsuitable for either of these requirements.

HAS LETHARGIC ENCEPHALITIS ANY RELATION TO EPIDEMIC PAROTITIS?

EDVARD GUNDERSEN

From the Army Bacteriological Laboratory, Oslo, Norway

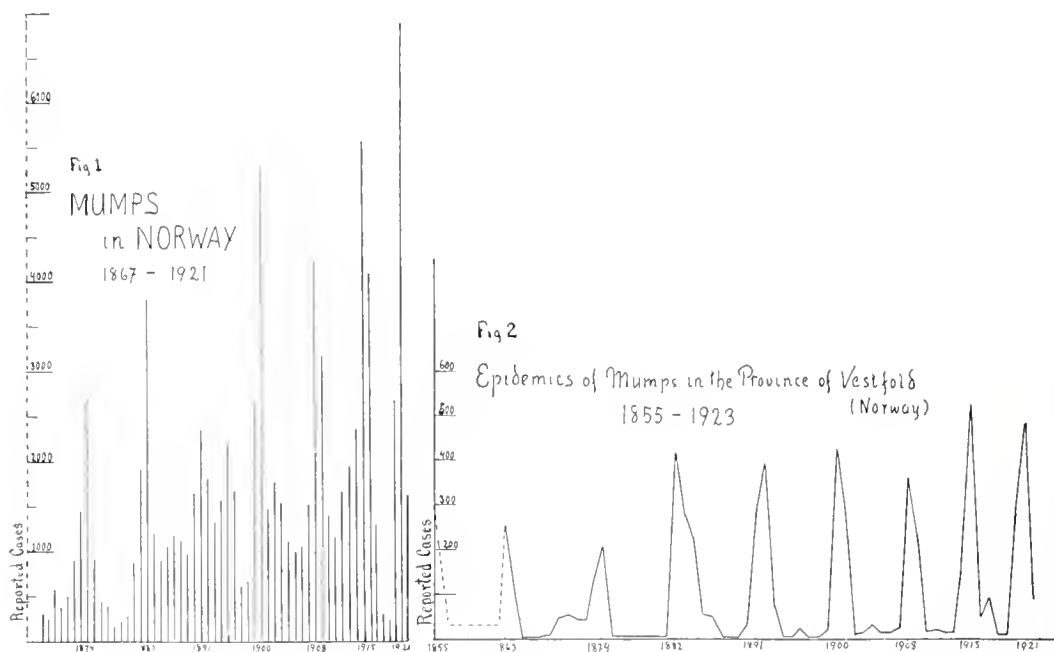
The etiology of lethargic encephalitis has not yet been made clear. There is, however, no doubt that the disease is infectious and that it is epidemic in spite of its sporadic occurrence. The trait which characterized it in Norway was the suddenness of its advent after its appearance during previous years in Central Europe. Most of the cases occurred during the winter months, distributed at various points and the morbidity was low.

The clinical peculiarities of lethargic encephalitis led to the belief that it was a specific disease. But the peculiar way in which the cases were distributed, their isolated occurrence here and there and their comparatively small number led to the supposition that the disease was a particular manifestation of some evenly distributed infection and the question of the relation of encephalitis to influenza arose. But this relation seemed to be very artificial. In Norway, cases of "sleeping sickness" occurred one or two years after the cessation of the influenza epidemic while in Central Europe it was observed before the influenza. Nor did influenzal encephalitis show the same clinical peculiarities as lethargic encephalitis. And furthermore from the point of view of pathologic anatomy it has been maintained that these were two different diseases. Thus the question is still unsettled whether lethargic encephalitis is a specific disease or whether it is related to other infectious diseases which recur in epidemics.

In connection with a study of the occurrence of epidemic parotitis in Norway during the last 100 years the author has dealt with the nervous complications attending parotitis and has discussed the possibility of some relation between it and lethargic encephalitis. So far as the author is aware this question has not been considered before and he therefore thinks it of some interest to deal with it here. As a point of departure it is necessary to review briefly the occurrence of epidemic parotitis in Norway.

Our medical reports mention parotitis as far back as the year 1834, but figures are not given until 1864. Cases of parotitis are always to

be found in this country. The disease may occur sporadically for a long series of years and then suddenly appear as an epidemic all over the country. The peculiar thing about these parotitic epidemics is that they occur at regular intervals. A parotitic epidemic is mentioned for the first time in 1834-1835 and they recur in 1845-1846, 1854-1856, 1863-1864, 1873-1874, 1882-1883, 1890-1891, 1900-1901, 1908-1909, 1915-1916 and 1920-1921. Thus parotitis returns as an epidemic at intervals of 8 or 10 years. Only in the case of the last two epidemics has the interval been shorter—6 to 7 years. In the years of parotitis we



find the disease spread all over the country as an epidemic. The same points of culmination are found in all the provinces. This is beautifully represented by the curve for the province of Vestfold (Norway). Here the periodicity is so marked that at any given time it should be possible to say when the next epidemic will appear. The distinctly pronounced periodicity of epidemic parotitis is a hitherto unknown trait in the epidemiology of the disease. Another important thing to note about epidemic parotitis is that the epidemics are distinctly winter epidemics. A comparison of the 10 largest epidemics in Oslo and Bergen shows that all of them culminated in December and January. They commence in October and November and come to an end in April and May. The extent of the infection during such an epidemic and the number

of persons attacked may be understood from a description of the epidemic in Lödingen and Steigen in 1899 by H. Arnesen. At the census in the following year an investigation showed that 25% of the population had had parotitis.

A study of the reports on these epidemics gives an impression of the varying forms in which the disease manifests itself in different persons. The same applies also to the different epidemics. Each one has its own characteristics. An epidemic may be mild, attack mostly children and consist of ordinary parotitis only. Another may attack sexually mature persons, be accompanied by violent fever and evince a strong tendency to metastasis effecting the testicles, ovaries and pancreas. In one epidemic cutaneous symptoms are in evidence, in another the symptoms are found in the central nervous system. Still other epidemics are characterized by symptoms in the mucous membranes, by gastric, bilious disturbances, or by symptoms in the urogenital tract. All in all it is found that the behavior of these epidemics is polymorphous.

Of the manifold varieties of symptoms manifested by this infection the symptoms of the central nervous system are here of most interest to us but we have least knowledge of them. We meet these cases of cerebral parotitis in the years when the epidemics are violent and at the time of culmination. The symptoms in the central nervous system may be of a more general character. They may manifest themselves as focal symptoms of the brain. Among the former cases status typhosus, observed during most of the epidemics, is most in evidence, accompanied often by delirium and convulsions. Or a markedly sporous condition may be found. In other cases parotitis may be accompanied by insomnia of long duration or followed by vertigo of long duration complicated sometimes by sudden deafness. In some cases parotitis causes maniacal attacks and in others chorea. While these manifestations of parotitic infection from the central nervous system are difficult to classify as a definite form of disease, parotitic meningitis, parotitic encephalitis and neuritis are more distinctly pronounced conditions.

It is not more than about 20 years ago that these peculiar manifestations of parotitis were first closely investigated. But in our medical reports we find these conditions mentioned, though sparingly, a long way back.

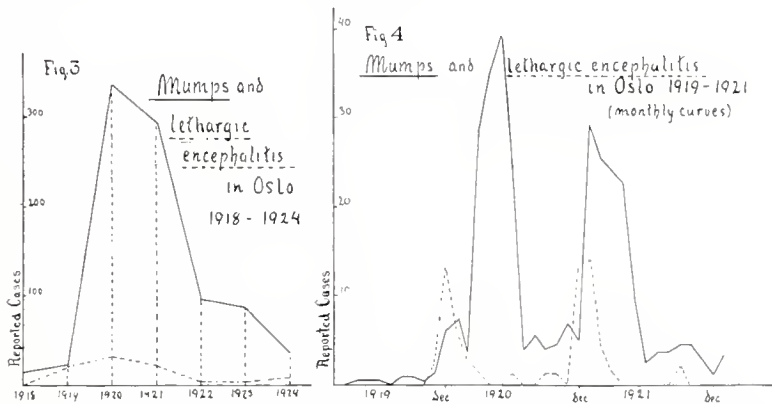
Skjelderup and Conradi each mentioned in the Medical Society in 1864 a case of parotitic encephalitis in connection with the parotitic epidemic then in progress in Oslo. In one case the power of speech was lost and the other was accompanied by sopor and brachycardia. In 1875 a report from Ibbestad mentions

three cases of apparently primary parotitic meningitis in a family of children without fatal parotitis while three other children in the same family had ordinary parotitis. In 1890 Kolbjørnsen mentions a case of parotitic meningitis accompanied by paresis. This parotitic meningitis is mentioned by Laache in his work "On Pseudomeningitis and Meningitic Conditions" and he there also refers to cephalalgia post parotitidem which is not of rare occurrence. It was not until the beginning of this century that this aspect of parotitic infection was made clear in work by the French—first and foremost by the work of Chauffard and Boidins. They have shown that meningitic affections are frequent in cases of parotitis.

This parotitic meningitis must be separated from the serous meningitis group as a septic amicrobic serous meningitis. While parotitic meningitis both clinically and as far as alterations of the arachnoid fluid are concerned is comparatively clear-cut, great lack of uniformity is met with in the forms of reaction from the central nervous system in the case of parotitic encephalitis. The same conditions as in infectious encephalitis are met with. While its meningitic equivalent has the stamp of uniformity, the encephalitis is met with in a great variety of forms. Also in the case of parotitic encephalitis it is necessary to speak of the complex of symptoms and what has been already referred to as cerebral complication in parotitis, cramp, paralysis, delirium, maniacal attacks, insomnia, depression, somnolence, vertigo, chorea, aphasia, deafness, etc., must in many cases be taken as an expression of this encephalitic process.

A great variety of infectious diseases may be accompanied by encephalitic complications which manifest the same pathologic-anatomical alterations. It is the degree of these and the localization of the foci of inflammation that determine the clinical forms of encephalitis. When it is a question of infectious diseases with an unknown etiology there are no means of separating the various forms of encephalitis, as for instance, primary parotitic encephalitis and infectious encephalitis. There is no tie to bind the etiologically uniform cases together. What now determines the relationship is the epidemic behavior. In the case of parotitic meningitis and encephalitis, parotitis is the common factor. But apart from the accompanying parotitis we may draw up from the medical reports the rough outline of the behavior of these cases of meningitis and encephalitis. They accumulate in certain years, the years of epidemic parotitis, and thus occur within a very limited space of time and the manner of their occurrence is sporadic, the infectious nature of the disease being, however, manifest.

In the great majority of cases the parotitic infection manifests itself only in primary parotitis. Same may then be accompanied by secondary orchitis, oöphoritis, pancreatitis, meningitis and encephalitis. But it may be seen that parotitic infection may manifest itself in primary orchitis without parotitis and in primary oöphoritis and pancreatitis. One would then expect that primary localization of parotitic infection in the brain without accompanying parotitis should also be possible. But if such localization of the parotitic virus ever occurs in these organs, as for instance the brain, we have hitherto at all event lacked means of knowing that the diseased condition has any connection with parotitis and the illnesses are therefore either classed as separate and distinct groups or they are classed together with other well-known diseases the symptoms of which manifest themselves in the central nervous system.



Such forms of primary parotitic meningitis and parotitic encephalitis would like secondary parotitic meningitis and encephalitis reappear within limited and sharply defined periods of time and they would likewise within these periods appear sporadically but nevertheless in such a manner as to be characterized as epidemic. Do then such cases of meningitis, or encephalitis e parotitide sine parotitide, occur?

In its epidemic form infectious myeloencephalitis behaves in the same way as parotitic meningitis and encephalitis. In this connection Tschudi-Madsen makes use of a striking analogy in his work on encephalitis lethargica, comparing these forms of encephalitis and the conditions of disease dependent thereon to islands, while under the surface, removed for the most part from our knowledge, is a continuous bottom which binds the whole together.

Figure 3 shows the occurrence of parotitis and sleeping sickness in Oslo during the period 1919-1924. Here the peculiar fact will be

noticed that it is just in the period when lethargic encephalitis occurred that the worst epidemic of parotitis was in progress judging from the available figures. It will be seen that the curves showing the occurrence of the two diseases in Oslo are parallel. They rise, reach a climax and fall again in exactly the same manner during this period.

This congruity between the two curves is still more in evidence when a monthly curve is plotted for the two diseases. Previous epidemics of parotitis have run their course during a single winter. Here we see that the epidemic bursts out again the following winter. The parotitic epidemic commences in December 1919 and at the same time the encephalitis curve rises and reaches the climax in December and January. The same relation between the two diseases is rediscovered in the following year. Now, it may be asked whether the parotitic epidemic is really the continuous bottom from which all the diffused cases of encephalitis arise? Or, is lethargic encephalitis to be considered as a form of primary parotitic encephalitis?

In addition to simultaneous occurrence a common trait of the two diseases was that they were both typically winter illnesses. Moreover, in its epidemic behavior and topographic distribution the encephalitis bore the stamp of parotitic meningitis and parotitic encephalitis.

In the case of these two diseases the etiology of which has not yet been made clear it is of course impossible to seek for the causal interdependence. And in order to throw light on the matter we are still obliged to look for the relations between them.

This synchronous occurrence of parotitis and encephalitis should therefore be observable not only in Oslo or in Norway, but everywhere where infectious myeloencephalitis has occurred. Now the epidemic of 1920-1921 was by no means a local epidemic. It was spread all over the country, equally in the North and in the South. The author has not been able to obtain reliable information as to the nature and occurrence of parotitis in Europe during the period in which encephalitis was observed. It is, however, very unlikely that parotitis has occurred only in Norway during these years especially as its geographical distribution here reached from 50 to 71 degrees of northern latitude—a distance equal to the distance between Norway and Venice.

One would, moreover, if any connection exists at all, expect occasionally to meet cases of encephalitis combined with parotitis. Netter emphasizes that this combination of encephalitis and parotitis or pronounced salivation is not of rare occurrence. In such cases one is faced with the diagnostic difficulty of deciding whether the case in hand is

really one of encephalitis lethargica combined with parotitis or one of parotitic encephalitis.

If the two diseases are interdependent cases of encephalitic condition should be discoverable in the previous epidemics of parotitis. It should here be borne in mind that the various epidemics were, on comparison, found to bear a very different stamp. The polymorphous characteristic of the parotitic epidemics should be a warning that one should by no means expect to rediscover the peculiarities of one particular epidemic in all the others. There is also another circumstance to be taken into consideration. We have seen that during nearly 100 years there has been a regular interval of 8 or 10 years between the years of parotitic epidemic. We found an exception in the case of the epidemic of 1920-1921, the interval being 5 or 6 years. This circumstance alone may indicate greater activity, greater virulence in the parotitic infection, which leads to this epidemic being characterized by the occurrence of relatively many cases of primary parotitic encephalitis or infectious encephalitis.

If one endeavor to trace the infectious encephalitis from the foregoing period it will be found that a thick veil is spread over its way, because it is only of late years that it has been possible to gather the manifold aspects of encephalitis together. These cases will in the reports be found classified under various groups of disease, typhoid fever, cerebral fever, inflammation of the brain, cramp, paralysis, etc.

The author has searched the medical reports for information as to the peculiarities of these cases which have been classified as typhoid fever and inflammation of the brain and noted whether they have occurred in the marked years of parotitis.

With our present knowledge of serous meningitis some forms of which seem to come etiologically under the heading of infectious encephalitis it is natural to search for the medical reports of these diseases under the heading, meningitis. The meningitis groups as well as the typhoid fever group must—in the period to be investigated—be looked for under the common diagnosis which includes, *inter alia*, both epidemic cerebrospinal meningitis and serous meningitis.

In 1835-1836 when the parotitic epidemic was in progress all over the country, mention is found in the reports from the Province of Bergenhus South under the heading of typhoid of a peculiar diseased condition observed widely in the district and manifesting itself partly in fits of fury and convulsions, partly in a paralytic condition of the brain and pronounced somnolence. Also at that time this disease was generally called "sleeping sickness."

During the next big epidemic in 1845 which raged especially in the Province of Buskerud Dr. T. Fr. Blich in Drammen reported that inflammation of the brain seemed to occur frequently either as a specific disease or as a complication of other diseases but he was not able to give any reason for this.

The next wave of parotitis comes in 1854-1855. In 1854 the disease was most widespread in the Eastern provinces. In that year it is reported from Ödemark and Rödenes, where the parotitic epidemic raged, that during the early days of the year some cases of cerebral typhoid of a very violent character ending in death within a few days occurred.

In 1855 parotitis was more widespread in the town of Skien than had previously been observed and in many cases it evinced meningitic symptoms. And at the same time J. A. Vetlesen reports that a greater number of cases of meningitis were observed than ever before.

The next wave of parotitis came in 1864-1866. It is from this period that Skjelderup's and Conradi's cases of parotitis combined with encephalitic phenomena date and it is just in this period that epidemic cerebrospinal meningitis bursts into activity especially in the Parish of Kristiansand and the Province of Romsdal. Is there any relation discoverable between these cases of meningitis and parotitis? As far as the country at large was concerned the epidemic was practically at an end in 1865. It was only in the district of the town of Mandal that the epidemic of parotitis was still in progress in 1865-1866. And it is just in this district that we see cases of meningitis suddenly appear in the period in question. Dietrichson says that the disease in this district in 1865 was plainly of rheumatic origin because it often turned into febris rheumatica. The symptoms most in evidence were pains and stiffness in the throat and back, violent headache and delirium, marked somnolence and brachycardia. From the same source we have mention of the observation in 1866 of similar cases combined with sopor, dorsal pains and brachycardia. The individuals attacked were as a rule under the age of 15 years.

The next great epidemic of parotitis commenced in 1873 and culminated in 1874. The disease was most widespread in the Eastern Provinces. The only place from which parotitic meningitis is reported is the Province of Kristiania. The following year cerebrospinal meningitis burst out in the Northern Provinces. From 1875 we find mention of the cases of primary parotitic meningitis in Ibbestad, already referred to, and arising from the epidemic of parotitis in progress during the Lofoten fishing season.

The parotitic epidemic of 1882-1883 was on the whole mild. Cases of cerebrospinal meningitis were only sporadic during these years. Only in the Province of Nordland was there an accumulation of cases, an epidemic of mild meningitis in Mo in Ranen. At this time parotitis was, however, very widespread in the Province of Nordland from Ranen to Lofoten East, so the possibility exists that these were cases of serous meningitis dependent on parotitic infection.

In 1886 parotitis appeared in the Province of Trondhjem South and took the form of a widespread epidemic. During the same year cerebrospinal meningitis appeared in the same place. In this part of the country there had not been many cases of this disease since the previous epidemic of parotitis in 1877.

The parotitic epidemics of the nineties were not so widespread as the previous epidemics and they were comparatively mild. In 1894 parotitis appeared in the Province of Romsdal and at the same time an increase took place in the number of cases of meningitis reported from that part of the country.

The next big epidemic of parotitis commenced in 1899. It culminated in 1900 and receded in 1901. This epidemic was very widespread indeed. Just during these years the meningitis curve rises for the whole country. The epidemic of parotitis commenced in the Provinces of Östfold and Akershus. In the Province of Östfold the first cases of meningitis appeared.

The coincidence between parotitis and meningitis already shown was characterized, *inter alia*, by appearance generally in the winter months being a com-

mon trait of the two diseases. In 1899-1900 the Province of Trondhjem South shows the greatest increase in the number of cases of meningitis. In the city of Trondhjem the greatest number of cases of meningitis occurred in the period May to July. And the epidemic of parotitis also shows the peculiarity of culmination in May.

From the epidemics of 1908 and 1915 practically no cerebral cases are reported. The disease seems to have been mild everywhere and there is nothing definite to go by in respect of its relation to the cases of meningitis occurring during these years.

In 1920 the last wave of parotitis swept over the country. At the same time infectious encephalitis appeared, not in the wake of the parotitic epidemic but riding on the top of the wave.

It may be that the relation intimated here between the epidemics of parotitis and certain peculiarities evinced by meningitis and encephalitis depends on something more than mere chance. It must be remembered that the diagnosis of cerebrospinal meningitis as a specific disease is of comparatively recent date. Before the establishment of the nature of meningitis by bacteriological means was possible and in view of the complicated representation of cerebrospinal meningitis given by the reports, it is permissible to doubt that the diagnoses are unshakable, especially when the mortality has not been in anything like reasonable proportion to the number of cases. It is likely that many of these cases have been serous meningitis because before the days of lumbar puncture epidemic cerebrospinal meningitis as a diagnostic group must have included cases of serous meningitis. Thus it is possible that we have been following the trail of serous meningitis. We are able to go no further.

It is to no purpose to endeavor to trace the encephalitic processes in the reports. We should be lost in a labyrinth.

SUMMARY

The epidemiologic peculiarities of lethargic encephalitis give reason to suppose that this disease is only a special form of manifestation of a more widely spread infection. The author concludes from a study of the epidemic occurrence of mumps in Norway during the last 100 years that there is reason to look upon this infection as the origin of lethargic encephalitis.

Epidemics of mumps recur regularly every 8 or 10 years. During these epidemics the occurrence of secondary encephalitis and secondary serous meningitis is observed and occasionally primary parotitic meningitis without mumps occurs.

Lethargic encephalitis occurred in Oslo in 1919 during an epidemic of mumps. The monthly and yearly curves for the two diseases show a striking parallelism. The epidemiologic peculiarities which characterize lethargic encephalitis are also to be found in encephalitis and meningitis of mumps. They show within a limited space of time isolated foci of infection, that however must be looked upon as parts of a prevailing epidemic. They are all typical winter diseases and they show the same topographic distribution. And clinically we find on one hand the combination of mumps with secondary encephalitis or on the other hand encephalitis lethargica with parotitis.

Upon investigation of the previous epidemics of mumps the author has found in these definite years before the time of lumbar puncture that the statistical reports of the occurrence of cerebrospinal meningitis and the cerebral and typhoid fevers show certain facts which make it reasonable to think that some of the epidemics of these diseases have not been what they are called in the reports, but in reality belong to the infection of mumps. And—during the last epidemic of mumps we again met these cerebral affections, but in a form now better known clinically than before, i. e., the lethargic encephalitis and its equivalent, serous meningitis.

FURTHER OBSERVATIONS ON THE SPECIFICITY OF THE GREEN-PRODUCING DIPLOCOCCUS IN MEASLES

RUTH TUNNICLIFF

From the John McCormick Institute for Infectious Diseases, Chicago

In previous articles ¹ I showed that green-producing, bile-insoluble cocci peculiar to measles, were present in largest numbers in the blood of measles patients before the appearance of the rash and diminished in number when the rash began to fade. Similar cocci were found in eye, nose, throat and sputum early in the disease and disappeared with the abatement of the catarrhal symptoms. The injection into a rabbit of a strain of green-producing cocci isolated from a measles patient as the rash was fading produced neither Koplik spots nor rashes, while rabbits given injections with strains isolated at the onset of an attack of measles, showed Koplik spots, rash and rise of temperature. It was also shown that strains of green-producing diplococci isolated at the onset of measles produced reactions in the skin of normal rabbits but not in those immune to measles, while strains isolated from measles late in the disease gave reactions in both normal and immune rabbits.

Strains of green-producing cocci isolated from measles patients during the disease have been tested with the serum of goats immunized with measles cocci (which were isolated before and during the appearance of the rash), and the green-producing cocci isolated late in the disease were found to be different from those specific for measles. The opsonic method was used in making these determinations.

Park, Williams and Wilson ² criticize the etiologic significance of green-producing cocci to measles because of finding similar cocci during convalescence when as a rule measles is known not to be contagious. On this account further experiments have been made with cocci isolated from the nasopharynx before the appearance of the rash and with cocci isolated during convalescence.

The following simplified opsonic method, which was found useful in identifying scarlet fever streptococci ³ and in testing the opsonic

Received for publication, June 30, 1927.

¹ J. Am. M. A., 1917, 68, p. 1028; 1918, 71, p. 104; J. Infect. Dis., 1918, 23, p. 572; 1922, 31, p. 382; 1923, 37, p. 193.

² Am. J. Pub. Health, 1927, 17, p. 460.

³ J. Am. M. A., 1926, 87, p. 625.

strength of serums from goats and horses immunized with measles diplococci, was used.

Normal and immune goat serums are heated at 56 C. for one-half hour, to get rid of normal opsonins. Some serums retain their opsonic content for months, and may be kept in the ice box for future use. It is necessary to dilute the serum 1:10 with salt solution if antiseptic has been added, to avoid inhibition of phagocytosis.

Equal parts of normal human blood and of sodium citrate solution (2% in physiologic salt solution) are mixed. Three large drops of blood are sufficient for twelve specimens. The citrated blood should be used soon after collected.

Cocci grown on blood agar for 24 hours are suspended in salt solution. Thick suspensions are to be avoided in order to escape getting too much phagocytosis for accurate counts. Only a slightly turbid suspension is desired.

Equal parts of serum, citrated blood and bacterial suspension are mixed in capillary pipets, and the mixtures incubated 25 minutes at 36 C. The mixtures are smeared on glass slides, 50 or more polymorphonuclear leukocytes counted and the number of cells taking part in phagocytosis noted (phagocytic index). If the phagocytic index of the immune serum exceeds that of normal serum determined by the same method, it is concluded that the coccus belongs to the measles group. These differences between the normal and immune specimens should be marked. The phagocytic index of the immune serum divided by that of normal serum represents the opsonic power of the immune serum. Strains of green-producing cocci from sources other than measles give indexes with immune serum varying from 0.55 to 1.0; those giving indexes from 2.0 up are considered to belong to the group of cocci specific for measles.

The green-producing cocci isolated from the nasopharynx of two patients before the appearance of the rash, and from two as the rash was beginning, were shown to belong to the group of cocci specific for measles. The green-producing cocci isolated from three of these patients nine days later in two cases and six days later in one, after the rash had disappeared, did not belong to the measles group. Similar cocci isolated from three other patients after the disappearance of the rash on the 7th, 10th and 14th days of the disease were shown not to be specific for measles. Green-producing cocci isolated in large numbers from the nasopharynx of a patient after the eruption had faded were found to be specific for measles (table 1).

Three patients whose rashes had faded showed no green-producing cocci in their cultures from the nasopharynx.

Two strains of green-producing diplococci isolated by Duval and Hibbard⁴ from the blood in the preeruptive stage of measles and two strains isolated by Cary and Day from the throat and blood of patients early in the disease have been found to belong to the group of measles cocci (table 1).

⁴ Proc. Soc. Exper. Biol. & Med., 1927, 24, p. 519.

These experiments indicate that while green-producing cocci may be present in the nasopharynx during convalescence from measles, in the majority of cases they are not specific for measles.

After reading Hadley's ⁵ article on microbic dissociation, it seemed interesting to try to determine if any environmental conditions had incited a dissociation of the measles cocci changing their immunologic reactions. A strain of measles diplococci was transplanted daily in one series of cultures of 10% normal horse serum dextrose broth and

TABLE 1

PHAGOCYTOSIS OF GREEN-PRODUCING DIPLOCOCCI ISOLATED FROM PATIENTS WITH GERMAN MEASLES, ACUTE RHINITIS, AND MEASLES AT DIFFERENT STAGES, IN MIXTURES WITH NORMAL AND IMMUNE GOAT SERUMS

Cocci From		Percentage of Leukocytes Showing Phagocytosis With Goat Serum	
		Normal	Immune (To Measles Diplococci)
Measles.....	Stage-with Reference to Rash		
Blood	Before.....	4	24
	Before (Duval and Hibbard).....	10	34
	(Duval and Hibbard).....	4	38
	At height (Cary and Gay).....	8	28
Throat	At height (Cary and Gay).....	10	33
	Beginning.....	0	52
Sputum	At height.....	12	46
Nose	Beginning.....	0	14
	Disappeared.....	20	46
	Before.....	0	16
	Disappeared.....	40	36
	Before.....	0	17
	Disappeared.....	26	20
	Beginning.....	4	24
	Disappeared.....	0	0
	Disappeared.....	26	20
	Disappeared.....	20	20
	Disappearing.....	6	12
	Disappeared.....	8	8
German Measles			
Blood.....		50	46
Acute Rhinitis			
Nose.....		9	5
Nose.....		48	40

in another series of 10% immune measles horse serum dextrose broth. The serums were furnished by Dr. Benjamin White of the Antitoxin and Vaccine Laboratory of the Massachusetts Department of Health. The Dochez method of immunization was used, employing the same strain of measles coccus that was used in this experiment. The serum had a high opsonic content for measles cocci. The cocci was transplanted from the broth culture to sheep blood agar and grown 24 hours. A salt solution suspension of these cocci was used in each experiment. The

⁵ J. Infect. Dis., 1927, 40, p. 1.

measles cocci retained their specificity during three generations in both normal and immune serum broth, but lost it by the seventh generation. There was no change in specificity when transplanted daily in dextrose broth. Cocci transplanted from the eleventh generation in normal horse dextrose broth, onto blood agar for four generations regained their specificity. The ninth generation on blood agar restored the specificity of cocci of the 12th generation in immune horse serum broth. These experiments indicate that specificity as determined by opsonic reactions, was lost in both normal and immune horse serum broth and could be regained by growing on blood agar for several generations. Why normal serum acted the same as immune serum was unexplained since the normal horse serum contained no opsonins, no antitoxin, no protecting bodies such as were present in the immune horse serum.

TABLE 2
EFFECT OF GROWING MEASLES COCCI AT DIFFERENT TEMPERATURES

Cocci Incubated		Percentage of Leukocytes Showing Phagocytosis with Goat Serum	
Hours	Temperature, C.	Normal	Immune
24	36	0	28
48	33	0	44
24	40	4	8
48	40	2	0
4 days	40	0	0

Since a rise of temperature generally occurs during an attack of measles and since, according to Hadley, temperatures higher than optimum have been known to change virulence and immunity reactions, the effect of different temperatures have been tried on the immunological reactions of measles cocci. The coccus grew well at 33 C. and at 40 and 41 C., but not at 42 C. Cultivation at 33 C. did not influence the coccus immunologically but growth at 40 and 41 C. completely removed its specificity as determined by phagocytability with immune goat serum (table 2). Their specificity was restored by transplanting again on blood agar and growing at 36 C. Three strains of cocci from measles were tested in this way and all lost their specificity when grown at 40 C. for 24 hours.

No change in morphology nor type of colony could be demonstrated coincident with the changes in immunity.

CONCLUSIONS

The green-producing diplococci isolated before the appearance of the rash in measles and during the acute stage of measles are immunologically distinct from nearly all similar cocci isolated during convalescence.

Measles cocci grown in normal and immune horse serum dextrose broth lose their specificity, as determined by the opsonic method. Their specificity may be restored by transferring them on blood agar at 36 C. for a few generations.

Growing measles cocci at room temperature does not affect their specificity, but growth at 40 and 41 C. completely removes their specificity as determined by the opsonic method.

EFFECT OF DRYING ON THE SPECIFICITY OF SCARLET FEVER STREPTOCOCCI

RUTH TUNNICLIFF

From the John McCormick Institute for Infectious Diseases, Chicago

In 1921 Dr. W. J. Matousak¹ isolated strains of hemolytic streptococci from rooms, eating utensils and attendants of scarlet fever patients. The hemolytic streptococci isolated from eating utensils and from the face mask of the nurse in attendance were found by agglutination and opsonic tests to belong to the scarlatinal group of streptococci; while those from the floor, the wall and the nurse's shoe, did not. No explanation could be found for these immunologic differences. The discussion by Hadley² on the effect of environmental conditions on changes in the immune reactions of organisms suggested that the immunologic differences noted in these experiments and in certain work of my own might be due to drying, since drying appeared to be the only environmental difference between streptococci on eating utensils and face masks and those on the floor.

The effect of drying scarlet fever streptococci without killing them was tried and the influence on their phagocytability was tested. It was found that scarlatinal streptococci could withstand drying at least eight weeks in a test tube. At this time the number of viable cocci was considerably diminished. The cocci were grown 24 hours in one-half cc. of phosphate broth which was then put in small tubes and centrifugated; the supernatant fluid was removed and the cocci dried at room temperature. After the cocci were dried, one-half cc. of phosphate broth was added and a loopful transferred to a blood agar slant and incubated 24 hours. A suspension of this growth in salt solution was used in the tests. The cocci were tested for their specificity by an opsonic method previously used in identifying scarlatinal streptococci.³ The immune serum was furnished by Dr. John F. Anderson of E. R. Squibb and Sons and was from a horse immunized with scarlatinal streptococci by the Zinsser-Grinnell blood clot method, recently described by Anderson and Leonard.⁴ The serum had a high opsonic content and was specific for

Received for publication, June 1, 1927.

¹ J. Infect. Dis., 1921, 29, p. 91.

² Ibid., 1927, 40, p. 1.

³ J. Am. M. A., 1926, 87, p. 625.

⁴ J. Immunol., 1927, 13, p. 365.

scarlet fever streptococci. After six months in the ice box it still retains to a high degree its ability to cause phagocytosis of scarlet fever streptococci.

Method.—Normal and immune horse serum are heated at 56 C. for one-half hour, to get rid of normal opsonins. If the serums contain antiseptics it is necessary to dilute them 1:10 with salt solution on account of the antiseptics inhibiting phagocytosis.

Equal parts of normal human blood and 2% sodium citrate in salt solution are mixed. Three large drops of blood are sufficient for twelve specimens. Whole blood instead of washed leukocytes has the advantage of containing fresh serum which activates the opsonins in the immune serum if that serum has not been recently collected. The citrated blood should be used soon after drawn.

TABLE 1

INFLUENCE OF DRYING IN TEST TUBE ON THE SPECIFICITY OF SCARLET FEVER STREPTOCOCCI AS SHOWN BY OPSONIC TESTS

	Percentage of Leukocytes Containing Dried Streptococci		(Control) Untreated Streptococci	
	Normal	Antistreptococcic	Normal	Antistreptococcic
Dried in test tube				
24 hours.....	0	2	0	22
5 days.....	4	2	2	18
5 weeks.....	0	0	0	12
24 hours, then on moist blood agar				
3 generations.....	4	4	2	18
6 generations.....	1	4	0	22
13 generations.....	2	0	0	20
24 generations.....	2	6	2	24
37 generations.....	8	8	2	16
45 generations.....	6	8	2	27
51 generations.....	12	51	6	38

Cocci grown 24 hours on blood agar are suspended in salt solution. Thick suspensions are to be avoided in order to escape getting too much phagocytosis for accurate counts. A very slightly turbid suspension is desirable.

Equal parts of serum, citrated blood and bacterial suspension are mixed in capillary pipets, and the mixtures incubated 25 minutes at 36 C. The mixtures are smeared on glass slides, 50 or more polymorphonuclear leukocytes counted and the number of cells taking part in phagocytosis noted (phagocytic index). If the phagocytic index of the immune serum exceeds that of normal serum determined by the same method, it is concluded that the coccus belongs to the scarlet fever group. The differences between the normal and immune specimens should be marked.

Streptococci dried twenty-four hours lost their specificity as determined by the opsonic method (table 1). By daily transferring the dried cocci on moist blood agar, the specificity was restored after 50 generations.

It has been known that hemolytic streptococci transferred from time to time on blood agar retain their specificity for years. If the cocci from an old dried blood agar slant are tested with immune serum, they are found to be taken up under the influence of immune serum no more than with normal serum, but if transferred to moist blood agar the greater tendency to be phagocytosed with immune serum is restored. Scarlet fever streptococci were transferred daily on dried blood agar for eleven generations and then transplanted daily on moist blood agar. Their phagocytability has not been restored after 24 transfers on moist blood agar (table 2).

TABLE 2
INFLUENCE OF DRYING ON FLOOR, AND OF CULTIVATION ON DRY AGAR, ON THE SPECIFICITY OF SCARLET FEVER STREPTOCOCCI AS SHOWN BY OPSONIC TESTS

	Percentage of Leukocytes Containing Dried Streptococci		(Control) Untreated Streptococci	
	Normal	Horse Serums Antistreptococcic	Normal	Horse Serums Antistreptococcic
Dried on floor				
24 hours.....	6	8	4	26
5 days.....	2	4	0	20
Grown on dried agar				
1 generation.....	0	2	14	32
3 generations.....	4	4	0	22
6 generations.....	0	2	0	22
11 generations.....	6	8	0	20
11 generations, then on moist blood agar				
1 generation.....	0	0	0	12
11 generations.....	4	8	2	24
13 generations.....	2	0	0	22
24 generations.....	2	6	2	24

In order to reproduce if possible the conditions of the original observation that streptococci isolated from the floor of rooms occupied by scarlet fever patients did not belong to the scarlatinal group of streptococci, a 24-hour phosphate broth culture of scarlatinal streptococci was poured on the tiled floor of a room in the hospital. The floor was first washed with soap and water and dried. A swab wet in phosphate broth was drawn over this surface and a blood agar plate inoculated with this swab and incubated. This culture was sterile. Similar cultures were made daily after pouring the streptococcus culture on the floor and hemolytic streptococci isolated on blood agar plates and tested for their specificity. Streptococci remained alive on the floor for seven days. Tests made with cultures isolated after drying for 24 hours and five days, showed that the cocci were no longer specific for scarlet fever, as determined by the opsonic test (table 2).

Since no change in morphology and type of colony coincident with the change in immune reactions could be demonstrated, it is impossible to determine whether the change in the immunologic reactions was due to a dissociation of the streptococcus or not.

A few tests were made to determine whether drying influenced toxin production. Dried and normal scarlatinal streptococci were grown in phosphate broth, and skin tests were made with filtrates of the cultures at the end of 24 hours and four days. Toxins from both dried and normal streptococci produced about the same reactions. It was found that after growing four days in broth the dried coccus regained its specificity (table 3). The length of time used in producing toxins may change

TABLE 3
EFFECT OF GROWING NORMAL AND DRIED SCARLET FEVER STREPTOCOCCI IN PHOSPHATE BROTH

° Organism	Horse Serum	Percentage of Leukocytes Containing Streptococci Growth in Phosphate Broth		
		24 Hours	48 Hours	96 Hours
Dried.....	Normal.....	0	4	10
	Antistreptococcic.....	0	5	32
Untreated.....	Normal.....	0	0	2
	Antistreptococcic.....	12	18	32

their specificity and may account for the discrepancies between the opsonic and agglutination reactions and the toxin production. There appears to be no such close relation between toxin production and opsonification as there is between opsonification and agglutination.

H. M. v. Jettinar ⁵ has also found that the toxin production of dried scarlatinal streptococci remains unaltered.

CONCLUSION

Scarlatinal streptococci appear to lose their specificity through drying, according to opsonic tests. This characteristic seems to be stable for about 50 generations, after transferring on moist blood agar. The drying of streptococci may explain why streptococci isolated from walls and floors of rooms occupied by scarlet fever patients do not belong to the scarlatinal group of streptococci.

⁵ Ztschr. f. Hyg. u. Infektionskr., 1927, 107, p. 266.

IMPROVED AUTOMATIC DEVICES FOR TRANS-PLANTING CULTURES

W. M. WEINER

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

While conducting experiments relative to the pathogenicity of *B. aertrycke*, it became necessary to make long series of transplants at various time intervals. The need for an automatic transplanting apparatus was keenly felt, but because the necessity of having very accurately timed transplants did not seem great, various syphoning machines were tried. The result of these trials was the perfection of a simple, easily sterilizable one piece apparatus made entirely of glass which is described in figure 1.

The operation of this apparatus is exceedingly simple in that the entire set-up can be placed in the autoclave and after sterilization the syphon is made by air pressure applied at (2) figure 1. The graduated screw clamp (fig. 4) is adjusted to allow the medium to flow through at sufficient rate to fill the primary chamber (7) to the level (8) within the desired interval of time and the secondary chamber inoculated through (10) with a broth culture.

Should it be desired that the medium be kept outside of the incubator the apparatus may be separated at 3, the ends wrapped in paper and then be sterilized in two parts. In setting up the apparatus the tubing is placed through an opening in the incubator, the paper wrappings removed and the connection made aseptical with the aid of a flame.

The interval between transplants is controlled by the speed with which medium is allowed to syphon into the primary chamber. The velocity of flow is controlled by the clamp (5). On reaching the level (8) the entire contents of the primary chamber (7) are syphoned over into the secondary chamber (9), where the growth is diluted up to the level (12) and is again syphoned off down to the level (9a), leaving a residue in which growth is maintained until the next interval. Samples are collected in the tube (14) by simply inverting the t-tube.

For subsequent studies it was essential that an apparatus be used which would satisfy the following requirements: maintain uniform periods of growth throughout a long series; permit the accurate determination of growth rates; permit the syphon flask, without increased difficulties in setting up the apparatus, to occupy a place outside the incubator, since certain media change in viscosity on remaining in the incubator; and permit the use of a relatively small incubator.

Received for publication, June 9, 1927.

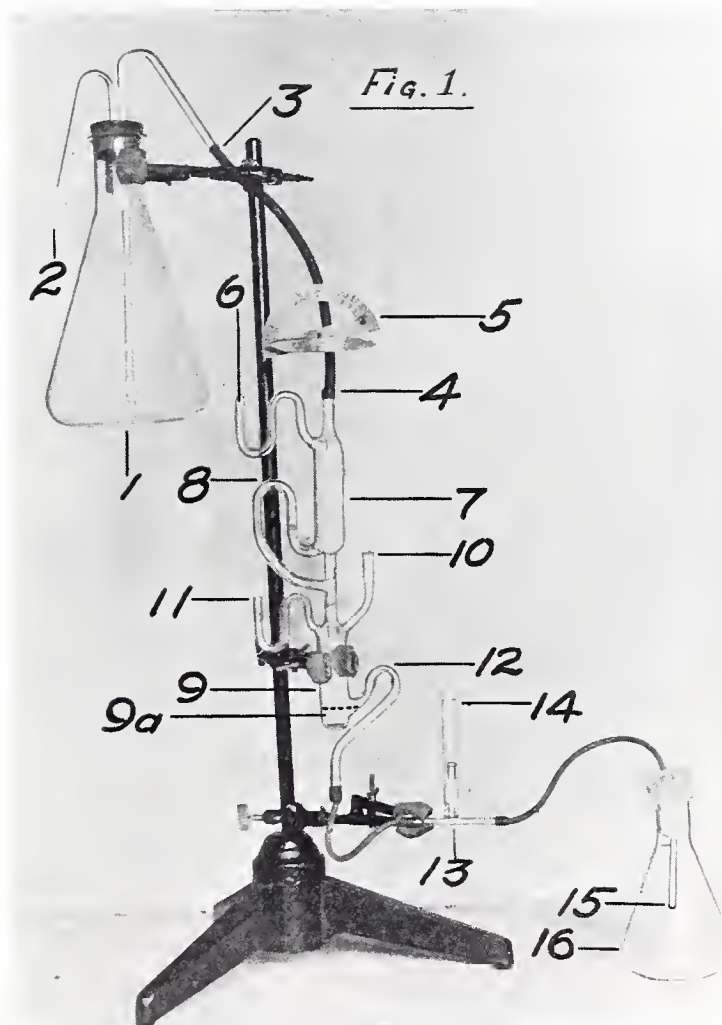


Fig. 1.—Automatic transplanting apparatus. 1.—Syphon flask containing medium. 2.—Air inlet. 3.—Junction of rubber tube with syphon tube. 4.—Junction of rubber tube with primary chamber. 5.—Graduated clamp (fig. 4). 6.—Air inlet. 7.—Primary chamber. 8.—Syphon level for primary chamber. 9.—Secondary chamber. 9a.—Growth level. 10.—Opening for inoculating chamber. 11.—Air inlet. 12.—Syphon level for secondary chamber. 13.—T-tube with opening to collect specimens in tube 14. 16.—Waste flask.

The apparatus of Felton and Dougherty¹ was considered but not procured since the possibility of its use for the work at hand was precluded by the following shortcomings:

The air inlet of the trap must extend to the level of the medium in the syphon flask, thus making the apparatus very difficult to set up with the medium outside of the incubator.

The flow of the medium depends on the release of a clamp on rubber tubing. The effect of repeatedly clamping rubber tubing is a loss of resiliency which results in less medium washing out the growth receptacle at each successive passage.

The impossibility of removing specimens at any desired time during the course of the growth studies precludes the determination of the growth curves of the various passages.

Specimens obtained by this machine come through tubing of greater length than permits reasonably accurate growth determination. The residue in this tubing, which has been incubated before, remains in the tubing in the horizontal position and mixes with the new material.

The apparatus illustrated in figures 2 and 3, constructed at a cost of approximately \$40.00, has satisfactorily fulfilled all of the above requirements for a period of several months. It may be deemed advisable briefly to explain the mechanism.

When the apparatus is set up as shown in figure 2 and a syphon is complete between the flask A and the trap E, the flow of medium is prevented by the plunger enclosed (fig. 3, A1). When an electric circuit is completed, a magnetic field is established about the trap E which raises the plunger contained within. Medium then flows into the growth chamber, diluting the contents and syphoning into the flask L, leaving a residue which extends only to the level H. It is this residue which constitutes the new transplant. Samples are collected at any desired time in the tube I by releasing the clamp at J. Following this, the tube I is removed and replaced by another sterile tube with the aid of a flame.

Operation.—The end of syphon tube (C) and the ends of both tubes (D) and (N) (fig. 2) are wrapped in paper. The entire apparatus is then sterilized in the autoclave in two parts.

After sterilization, the stand (O) (fig. 2) with primary and secondary chambers (E) and (H) are placed in the incubator, the solenoid (F) placed into position, the tube (D) passed through the top opening of the incubator, unwrapped and aseptically fitted to (C). The free end of the tube conducting waste is then unwrapped and aseptically placed into the mouth of flask L, which is then again stoppered.

¹ J. Exper. Med., 1924, 39, p. 137.

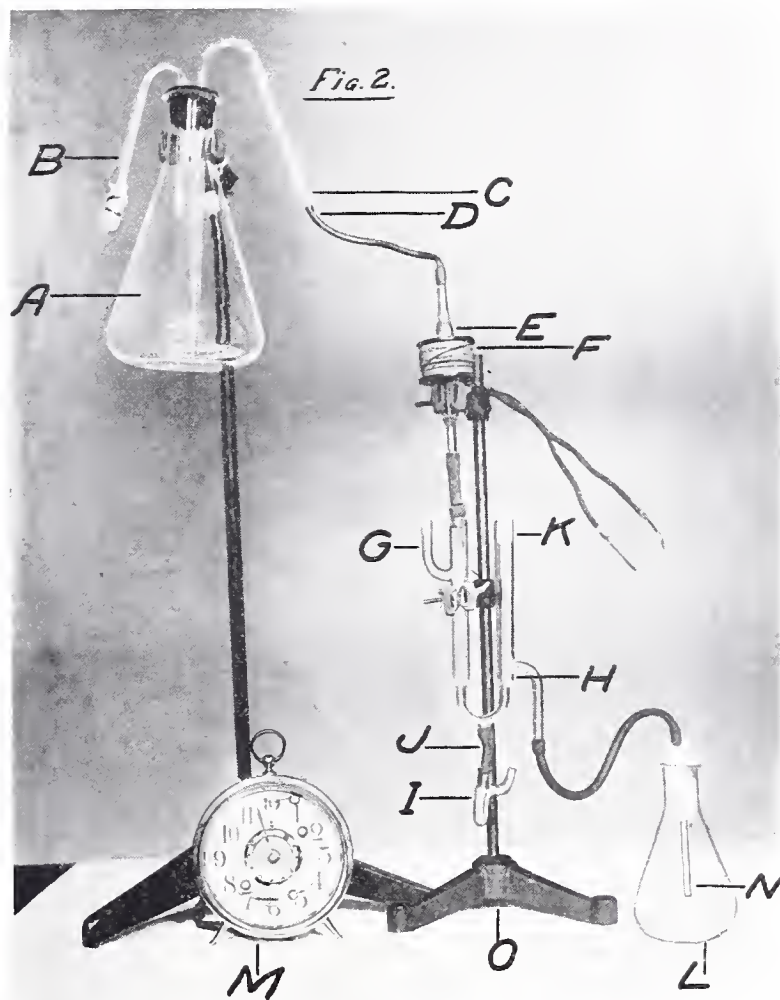


Fig. 2.—Automatic transplanting apparatus. A.—Syphon flask containing medium. B.—Air inlet. C.—End of syphon tube. D.—Tubing conducting medium to trap. E.—Trap containing plunger (fig. 3, A.). F.—Solenoid operated by clock and storage battery. G.—Air inlet to growth chamber. H.—Growth level. I.—Sample collecting tube. J.—Rubber tube (kept clamped when not emitting sample). K.—Opening for inoculating growth chamber. L.—Waste flask. M.—Clock acting as automatic switch (fig. 3, B).

After the apparatus is connected in this manner the tube J (fig. 2) is clamped with a screw clamp. The plunger (A1) of the primary chamber A (fig. 3) is raised by "shorting the clock out of the circuit." This may be done by connecting the points X and Y with a copper wire (see plan of circuits, fig. 5). Blowing gently into tube B (fig. 2) will establish the syphon and the wire used to establish the short circuit is immediately removed.

The clock is set by using the same method as in the case of an ordinary alarm clock. The disc is rotated until one of the lugs (B2) (fig. 3) comes in contact with the tungsten wire B6. Next the minute hand is placed into contact with wire B5, just before the eccentric disc B4 comes in contact with the silver pan B7.

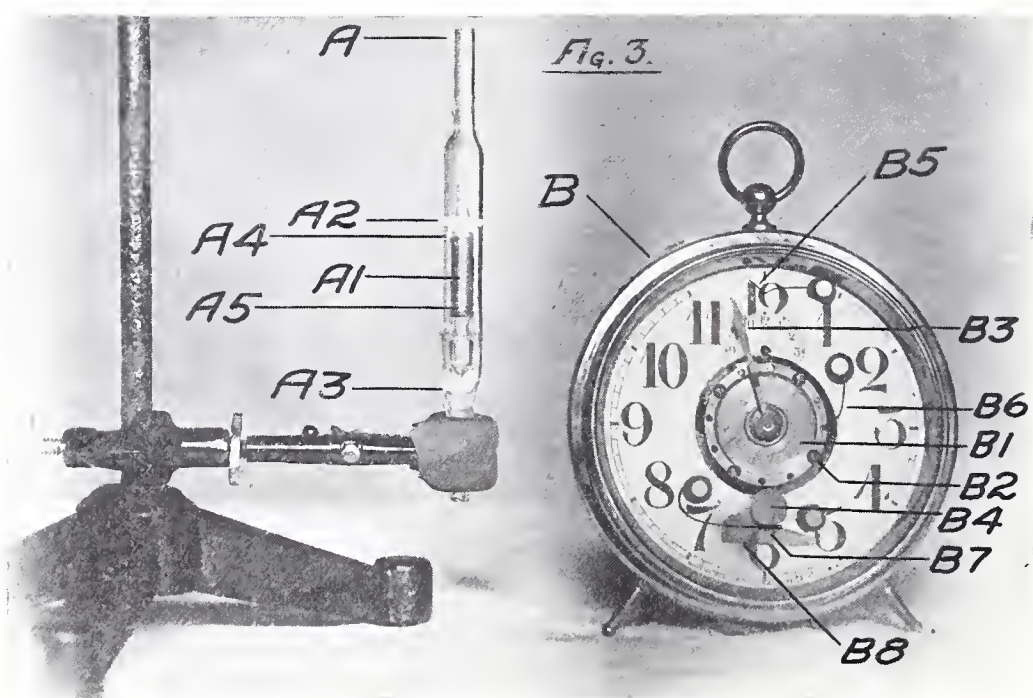
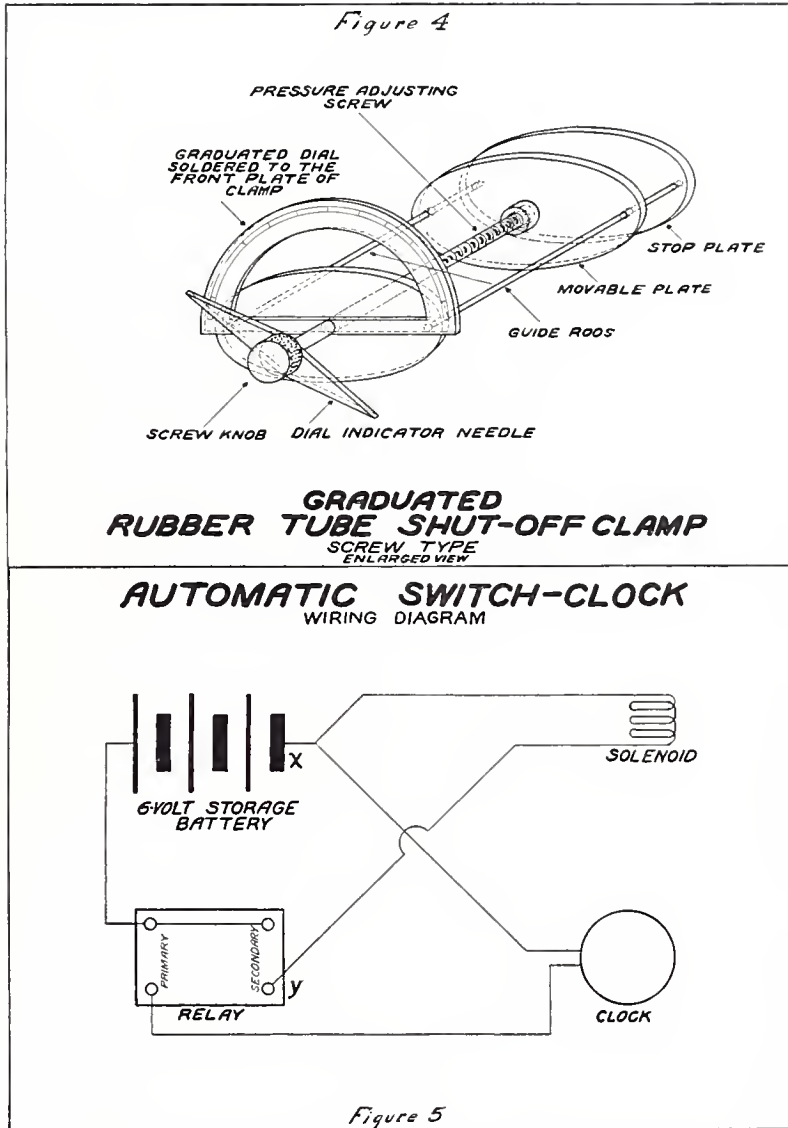


Fig. 3.—A.—Primary chamber (fig. 2, E). A1.—Plunger. A2.—Guides to prevent sticking of plunger. A3.—Ground glass joint for plunger. A4.— $1/64$ in. space between core and core seat. A5.—Pig iron core of plunger. B.—Clock used as switch (fig. 2, M): B1.—Copper disc replacing hour hand, with screw sockets for copper lugs. B2.—Copper lugs spaced at 3-hour intervals, each making contact with tungsten wire, B6, once every 12 hours. B3.—Minute hand making contact with tungsten wire, B5, once every hour. B4.—Eccentric copper disc soldered to second hand axis making contact with B7 once every minute. B5.—Tungsten wire contact for minute hand. B6.—Tungsten wire contact for lugs on disc. B7.—Pan on tungsten wire contact for eccentric disc, B4. B8.—Lever for adjusting B7.

When this last contact is established the circuit will be complete, the plunger in the primary chamber E (fig. 2) is raised by the magnetic field established by the solenoid and medium flows into the growth chamber H. The amount of medium thus flushing out H is regulated by moving the lever B8 (fig. 3). The apparatus is allowed to operate for 24 hours and the sterility of the broth in the flask L (fig. 2) is then tested.

The advantages of this apparatus are as follows: the constancy of the amount of medium carried through each time does not depend on the use of a head for equalizing the pressure, and the errors involved in clamping a rubber tube are avoided; the use of the sample



tube I coming directly from H permits the accurate determination of growth rates, provided care is taken to flush out the very short tube J after each passage; the ease with which the apparatus may be cleaned, sterilized and set up with small danger of contamination or breakage;

two or more apparatus may be run on the same circuit in the same incubator and thus results may be procured under comparable conditions; the greater accuracy of a standard clock as compared to any timing device using cogs, due to the continual slipping up of gears in constant motion; and the ease with which the flow of the medium may be regulated to maintain a flow for any period ranging between two seconds and a full minute is a very decided advantage in working with mediums of different viscosities.

SUMMARY

An automatic transplanting apparatus is described which permits the accurate determinations of growth rates of bacterial cultures, and is easily cleaned, sterilized and set up.

ACTION OF *B. WELCHII* TOXIN AND OTHER HEMOTOXINS ON ERYTHROCYTES IN VIVO

GUILFORD B. REED, J. H. ORR AND C. MARION SPENCE

From the Department of Bacteriology, Queen's University, Kingston, Canada

It has recently been shown by Cornell¹ that the injection of suitable cultures of *B. welchii* into rabbits produces a long standing infection accompanied by changes in the blood which suggest pernicious anemia. These observations were confirmed by the writers² and supplemented by a demonstration of the quantitative similarity of the anisocytosis in a series of inoculated rabbits with the anisocytosis in pernicious anemia in man. Kahn and Torrey³ have shown that the injection of *B. welchii* toxin into monkeys results in a blood picture like that of pernicious anemia. In our former paper it was also shown that rabbits which exhibited marked anemia following subcutaneous inoculation with *B. welchii* cultures usually suffered from strictly localized or spreading local infections and that other tissues, especially the blood-forming tissues, remained sterile.

The present paper offers quantitative evidence of the degree of anisocytosis in rabbits following the injection of *B. welchii* toxins and a comparison of these data with the results of the injection of other familiar hemotoxins.

B. Welchii Toxin.—The toxins used in these experiments were obtained from four strains of *B. welchii*: strain 1, which was used in our former work on infections, probably Bull's 911 strain; strain 2, a culture from the Lister Institute collection; and strains 3 and 4 which were recently isolated from the stools of patients with pernicious anemia. The cultures in each case were plated repeatedly in anaerobic jars to insure purity. For the toxin preparation the organisms were grown in the chopped beef-peptone medium recommended by Bengtson.⁴ The cultures were ordinarily inoculated with the breast muscle of a pigeon infected six to eight hours previously. After 24 hours at 37 C. the cultures were centrifugated and filtered through a Berkefeld N. candle or a small Steitz filter. The toxic filtrates were in every way similar

Received for publication, May 10, 1927.

¹ J. Infect. Dis., 1925, 36, pp. 425, 508.

² Reed, G. B., Orr, J. H., and Burleigh, C. H.: Canadian M. A. J., 1926, 15, p. 525.

³ Proc. Soc. Exper. Biol. & Med., 1925, 23, p. 8.

⁴ Hyg. Lab. Bull. 122.

to those described by other workers. The fatal mouse dose was from 0.25 to 0.5 cc., hemolysis of 5% washed rabbit red cells was effective in dilutions of the toxin of 1:100 to 1:500. Some preparations, especially those which filtered very slowly, were less active and were discarded. It is apparent therefore that there was no unusual activity in these preparations.

The following protocols are representative of the results with a considerable series of animals. The curves for variation of the red cells, referred to in the protocols were determined in the manner described in our former paper² and essentially similar to those determined by Price-Jones⁵ in the case of anemia in man.

TABLE 1
IN VIVO ACTION OF *B. WELCHII* TOXINS IN RABBITS

Rabbit Number	Toxin	Dose (MLD for Mice)	Time After Injection	Temperature, C.	Red Cells, Per Cmm.	White Cells, Per Cmm.	Hemoglobin, %	Weight, Gm.
1	<i>B. Welchii</i> , 1	10	(0, normal)	39.5	6,200,000	5,500	90
			1 hour....	40	4,300,000	2,700	80
			2 hours....	41	3,600,000	3,300	70
			4 hours....	41.5	2,400,000	4,400	65
			6 hours....	41	1,900,000	4,600	60
			7 hours....	Death in convulsions				
2	<i>B. Welchii</i> , 1	5	(0, normal)	40	5,140,000	9,100	90	2,280
			2½ hours..	41.5	4,600,000	11,000	83
			24 hours...	40.5	4,458,000	17,300	80	2,280
			48 hours...	39.2	4,370,000	11,280	80	2,160
			76 hours...	39.5	4,070,000	11,600	82	2,100
			5 days....	4,758,000	9,000	85	2,280
			10 days....	Animal normal				
3	<i>B. Welchii</i> , 2	4	(0, normal)	6,610,000	85
			2 hours....	4,780,000	77
			6 hours....	3,260,000	46
			1 day....	2,330,000	38
			2 days....	2,510,000	29
			4 days....	2,780,000	38
			5 days....	3,150,000	39
			10 days....	3,750,000	63
			17 days....	5,090,000	71
			26 days....	5,630,000	80
			Animal completely recovered					

Rabbit 1 was given 5 cc. of *B. welchii* toxin (strain 1) subcutaneously. This amounted to approximately 10 times the MLD for a 20 gm. mouse. The general effects of the toxin on the animals are summarized in table 1. It is apparent from the table that the red cell count fell progressively from the time the toxin was injected to the time of death. The red cell destruction was accompanied by a lesser percentage decrease in hemoglobin so that the index remained above one. Two hours after administration of the toxin when about half of the cells had been destroyed 50 cc. of urine were voided which consisted of a dark greenish-black fluid rich in free hemoglobin as shown by the gum guaiac reaction but entirely free of intact blood cells. Just before death a considerable feces, semi-fluid and greenish-black, was passed.

Curves of anisocytosis (fig. 1) indicate that soon after injection of the toxin most of the red cells were conspicuously smaller than the normal. Notwithstanding

⁵ J. Path. & Bact., 1922, 25, p. 487.

the progressive fall in the red cell count the new cells or those which escaped destruction were somewhat larger than in the initial stages of the toxemia.

Rabbit 2 was given subcutaneously 0.4 cc. *B. welchii* toxin (strain 1) amounting to 5 MLD for a 20 gm. mouse. The general effects on the animal are shown in table 1. In contrast with the reaction of the former animal which received about twice the dose of toxin there was only a slight decrease in red cells and an increase, rather than a decrease in white cells.

Anisocytosis curves (fig. 2) indicate that, as in the former case, the red cells which escaped destruction by the initial action of the toxin became largely microcytes. At the end of 24 hours though the count remained at approximately

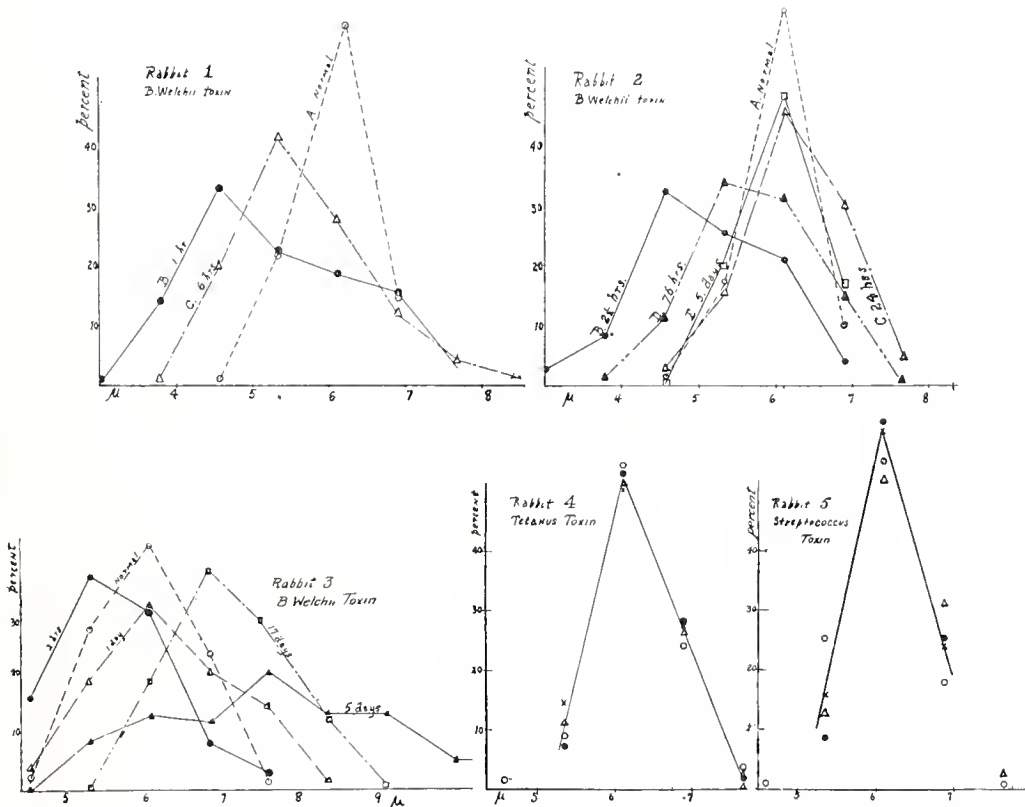


Fig. 1.—Variation in size of red blood cells in rabbit 1 following the injection of 10 MLD (for mice) of *B. welchii* toxin. Ordinates represent the % of the total number of cells counted and the abscissa the diameter of the cells in microns, in all figures. The dash line curve is for the normal animal before injection, the others are for the same animal at various intervals, as marked in the figure, after the injection of toxin.

Fig. 2.—Similarly the variation in size of red cells after injection of 5 MLD (for mice) of *B. welchii* toxin in rabbit 2.

Fig. 3.—The variation in size of red cells in rabbit 3 after injection of 4 MLD (for mice) of *B. welchii* toxin.

Fig. 4.—Variation in size of red blood cells in rabbit 4 following the injection of one-fifth MLD (for rabbit) of tetanus toxin for a similar adult rabbit. The points indicated by X are for the normal animal, before injection, those indicated by O, two hours; ●, one day; and △, two days after injection.

Fig. 5.—Variation in size of red blood cells in rabbit 5 following the subcutaneous introduction of *Streptococcus scarlatinae* by the agar block method. The points indicated by X are for the normal animal before inoculation, those indicated by O, two days; ●, four days; and △, seven days after injection.

the level attained in 2½ hours an appreciable number of macrocytes developed. By the fifth day the cells had approximately resumed their normal structure. The animal completely recovered.

Rabbit 3 was given 4 MLD for the mouse of *B. welchii* toxin (strain 2). The red cell count and hemoglobin determinations (table 1) indicate that there was a much more definite and prolonged red cell destruction than in the former case. The anisocytosis curves (fig. 3) indicate, however, the same tendency to cell size variation. But in this animal where the fall in red cell count was greater and prolonged, more and larger macrocytes were produced and at the same time more poikilocytes, nucleated red cells and polychromatophilic cells developed than were apparent in animals suffering less blood destruction.

Comparison of these results with those previously obtained with *B. welchii* infections show the two effects to be almost identical. In both cases the first perceptible effect on the blood is a decrease in the red cell count accompanied by the appearance of microcytes. If the dose of toxin is sufficiently large the animal dies with the blood in this state. But ordinarily with infections and with smaller doses of toxin the initial high number of microcytes is gradually replaced by more and more macrocytes, a change which is generally accompanied by a gradual increase in the red cell count. In the case of toxin injection the period of low count with microcytes predominating persists for only a few hours followed by death or by a rising count and increasing macrocytes, a condition which persists for several days. With infections the period of minimum count and predominating microcytes develops only after one to several days and the period of persistent low or rising count with conspicuous macrocytes may last for several weeks.

The latter period, in the case both of toxin injection and of infection, characterized by a stable or gradually increasing red cell count and conspicuous macrocytes ordinarily also exhibits numerous polychromatic cells, especially macrocytes, the maximum amount of poikilocytosis and usually a considerable number of nucleated red cells. The poikilocytes and nucleated red cells are most numerous in the cases where the anemia is most prolonged. This period in the reaction most closely resembles pernicious anemia in man.

Tetanolysin.—Tetanolysin has been prepared from a rather active toxin-producing strain of *B. tetani*. Anaerobic cultures were grown in a beef infusion-peptone broth and filtered after 24 hours of growth, when the concentration of tetanolysin appeared to be relatively high and of tetanospasmin relatively low though no exact comparison of the concentration of the two toxins has been made. The following case report is characteristic of the behaviour of several rabbits subjected to intravenous inoculation with these tetanolysin preparations.

Rabbit 4 was given intravenously one fifth of a minimum fatal dose of fresh tetanus toxin for a similar adult rabbit. The animal gave no indication of illness. The reaction of the blood is shown in table 2. The initial drop of 20% in the red cell count is similar to that shown in table 1 for *B. welchii* toxemia, but recovery from the tetanus toxemia was much more rapid. Films of the blood prepared at various intervals after the injection of toxin showed no perceptible departure from the normal structure. The results of a series of cell size measurements are shown in figure 4. It is apparent that there is no anisocytosis at any stage of the anemia. A similar degree of destruction of red cells by *B. welchii* toxin always resulted in conspicuous anisocytosis.

Streptolysin, Staphylolysin and Pneumococcus Hemotoxin.—Three actively hemolytic strains of Streptococci have been used: one isolated from a slightly inflamed throat, one from erysipelas, and a strain of *Streptococcus scarlatinae* isolated by Dick and Dick and obtained from

TABLE 2
IN VIVO ACTION OF TOXINS OF *B. TETANI* AND *STREPTOCOCCUS SCARLATINAE* ON
BLOOD CELLS

Rabbit Number	Toxin	Time After Injection	Red Cells, per Cmm.	White Cells, per Cmm.	Hemoglobin, %
3	<i>B. tetanus</i>	(0, normal)	5,370,000	71
		2 hours	3,870,000	54
		4 hours	4,270,000	59
		6 hours	4,930,000	55
		1 day	5,640,000	57
		2 days	5,470,000	57
		10 days	5,500,000	72
4	<i>Streptococcus scarlatinae</i>	(0, normal)	5,400,000	8,000	80
		1 day	4,930,000	77
		2 days	4,650,000	57
		4 days	3,720,000	22,400	58
		6 days	4,680,000	16,500	59
		7 days	3,480,000	11,200	56

the American Type Culture collection. An actively hemolytic staphylococcus isolated from a boil and a type 1 pneumococcus were used. All these cocci were grown in an infusion broth containing 1% rabbit serum, for 48 hours and centrifugated until a clear fluid was obtained. This clear fluid was injected into rabbits intravenously. Other rabbits have been infected with these cultures by Dochez's method of first injecting subcutaneously fluid agar and injecting a small amount of culture into the subcutaneous agar block.

The blood reaction of a rabbit to the toxin of *Streptococcus scarlatina* introduced by the infected agar block method is shown in table 2. The figures in the table indicate a very pronounced and persisting destruction of red blood cells. Notwithstanding the extent of the anemia, a careful study of blood films taken at intervals during the infection failed to show any definite departure of the red cells from the normal structure. Curves of the variation in size of red blood cells, shown in

figure 5, clearly indicate the complete absence of measurable anisocytosis. It should be noted, however, that a casual microscopic examination of such blood films often show cells larger or smaller than the normal but the relative number of the variants is small and the extent of the size variation slight as the curves demonstrate.

A fall in the number of red cells as great as that shown in table 2 for a streptococcus toxemia when brought about by infection with *B. welchii* or by *B. welchii* toxin is always accompanied by a conspicuous and readily measurable change in form and size of the red cells as demonstrated in the previous section.

The other strains of streptococci, staphylococci and pneumococci, mentioned earlier, when introduced into rabbits in agar blocks or when their germ-free culture fluids are injected, produce effects which are in every way comparable with those detailed for the *Streptococcus*. In other words, though these hemolytic cocci produced marked anemia in rabbits, the blood destruction is not accompanied by any conspicuous change in the size or the form of the erythrocytes.

CONCLUSION

It may be concluded from the data presented that the injection of *B. welchii* toxin into rabbits results in marked destruction of red blood cells, accompanied by definite variations in size. The injection of tetanolysin, staphylolysin, streptolysin and the hemotoxin of pneumococci in doses sufficient to produce an equally marked decrease in circulating red cells does not result in any measurable alteration in the size or the form of the remaining cells.

ACTION OF *B. WELCHII* TOXIN AND OTHER HEMOTOXINS ON ERYTHROCYTES IN VITRO

GUILFORD B. REED AND J. H. ORR

From the Department of Bacteriology, Queen's University, Kingston, Canada

In a former paper¹ we have shown that the injection of a *B. welchii* toxin into rabbits results in an anemia characterized by a decrease in red cells proportional to the dose of toxin, and accompanied by a marked anisocytosis and some poikilocytosis. Following the injection of the toxin, it was shown, there is a rapid decrease in the number of circulating red cells and a corresponding development of microcytes. Where the animal survives the initial toxemia for more than a few hours there is a gradual rise in the red cell counts and associated with it a replacement of the microcytes by macrocytes, some poikilocytes, much polychromatic staining and usually a good many nucleated red blood cells. These observations together with the fact that the most pronounced effects of a large dose of toxin are observable in a few minutes after injection have suggested that the action is a direct one upon the circulating red cells. Accordingly the action of *B. welchii* toxin has been tested on red cells in vitro.

Several methods of preparing red cells for treatment with toxin were tested. The cells were washed from defibrinated or citrated blood in various physiologic salt solutions with or without gelatin, as used by Brooks.² Though successful as far as protection from hemolysis was concerned we found that histologically these washed red cells were always to a greater or less extent distorted, the stained films always exhibited a considerable number of wrinkled, folded or crenated cells. We then resorted to the use of defibrinated blood with much better success. The blood was drawn from the rabbit heart and shaken very gently with glass beads, decanted from the fibrin and mixed with an equal volume of Tyrode's solution. Films from this preparation were indistinguishable from those made direct from drops of whole blood obtained by puncture of a vein. Moreover after ten to twelve hours incubation, considerably beyond the time that the experiments were carried, they retained their normal histologic structure.

¹ Reed, G. B.; Orr, J. H., and Spence, C. M.: *J. Infect. Dis.*, 1927, 41, p. 283.

² *J. M. Res.*, 1920, 41, p. 399.

In testing the action of toxins in vitro the desired dilution of the toxin was made with Tyrode's solution and equal volumes of it and the defibrinated blood combined. The controls consisted of Tyrode's solution and sterile broth in the same dilution as the toxin together with the defibrinated blood. The mixtures were incubated in 5 cc. amounts in rocking tubes * submerged in a water thermostat at 37.5 C. At intervals the degree of hemolysis was determined by centrifugating and comparing, colorimetrically, the clear fluid with standard solutions of the same blood laked in distilled water, the procedure used by Henry³ and similar to that of Brooks.² At the same time films were made of the blood, stained with Wright's stain and the form of the red cells carefully studied. Comparison of cell size under the action of different toxins or different degrees of hemolysis was facilitated by plotting size variation curves as for the experiments in vivo.

B. Welchii Toxin.—The effects of several samples of *B. welchii* toxin in various concentration have been determined. The rate of hemolysis of 50% defibrinated blood by a six weeks old sample of toxin in a dilution of 1:100 is as follows:

Time Minutes	Hemolysis %
12	2
15	3
20	10
30	20
50	30

The variations in the size of the red cells have been plotted (fig. 1) for each of the three degrees of hemolysis noted in the tabulation. It is apparent that after twelve minutes when 2% of the cells have been hemolyzed, the remaining 98% of intact cells are predominately microcytes and after fifty minutes when 30% had been hemolyzed the remaining cells were still smaller than the normal though not quite as small as in the initial period of the reaction.

TABLE 1
COMPARISON OF RATES OF HEMOLYSIS OF WELCHII, TETANUS AND STREPTOCOCCUS
HEMOTOXINS

Minutes	% Hemolysis Produced by Toxins of—		
	B. Welchii 1:200	B. Tetani 1:200	Streptococcus 1:40
80	1	2	5
150	7	4	10
240	20	5	20

The first column of table 1, and figure 2 show the results of a similar determination using a different sample of *B. welchii* toxin and using it in a much higher dilution. It is apparent from the table that this sample or this dilution of toxin produces a very much slower

* These rocking tubes consisted of L-shaped tubes of 6 cc. capacity in the lower limb of the L. They were supported on a rack, geared to a motor in such a way that the horizontal limb of the L containing the test mixture was tilted from end to end at a rate of 30 times per minute. This insured uniform mixing of the reacting substance.

³ J. Path. & Bact., 1922, 25, p. 1.

hemolysis, than in the former case. Examination of the curves in figure 2 also indicates that the development of microcytes among the cells which resist hemolysis is a progressive reaction. At 80 minutes, when 1% had been hemolyzed, the remaining cells were distinctly smaller than the normal but at 150 minutes when 7% had been hemolyzed the remaining cells were still smaller, considerably more than half the number being microcytes. At four hours, however, when 20% had been

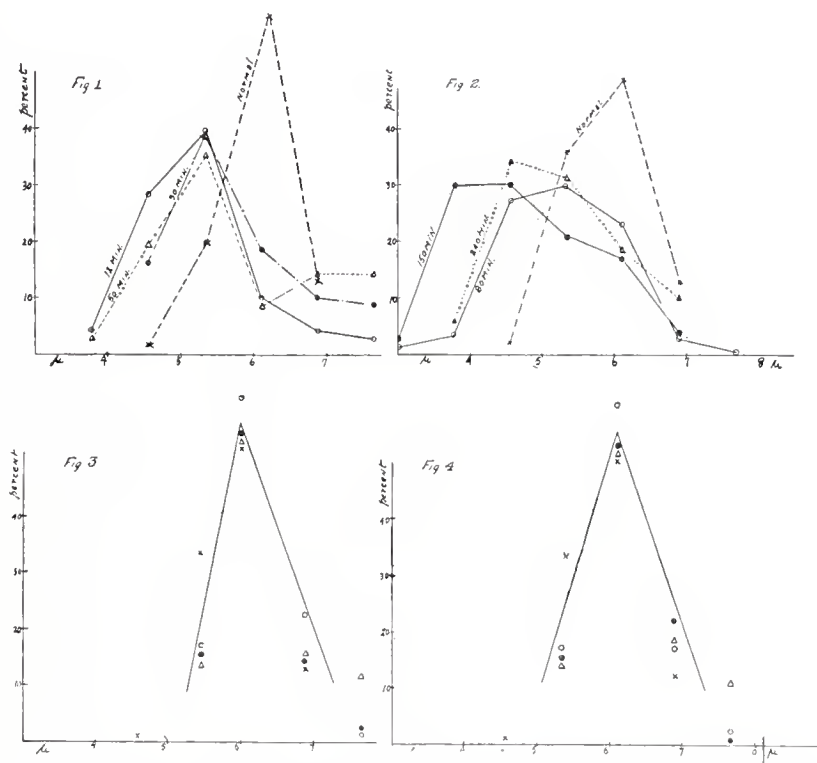


Fig. 1.—Variation in size of red blood cells treated with *B. welchii* toxin. Ordinates represent the percentage of the total number of cells counted, abscissas the diameter of the cells in microns in all figures. The — — — — curve represents the size variation of the normal untreated blood; — — — —, after 12 minutes' action of toxin when 2% of the cells had been hemolyzed; — — — —, after 30 minutes when 20% had been hemolyzed; - - - - - , after 50 minutes when 20% of the original number of cells had been hemolyzed.

Fig. 2.—Variation in size of red cells treated with *B. welchii* toxin. The — * — curve represents the size variation of the normal untreated blood; — ○ —, after 80 minutes' action of the toxin when 1% of the cells had been hemolyzed; — ● —, after 150 minutes when 7% had been hemolyzed; — — — —, after 240 minutes when 20% of the original number of cells had been hemolyzed.

Fig. 3.—Variation in size of cells treated with tetanus toxin. The points on the curve marked X represent the normal untreated red cells; ○, after 80 minutes' action of the toxin when 2% of the cells had been hemolyzed; ●, after 150 minutes when 4% had been hemolyzed; △, after 240 minutes when 5% of the original number of cells had been hemolyzed.

Fig. 4.—Variation in size of red cells treated with streptococcus hemotoxin. The points on the curve marked X represent the normal untreated red cells; ○, after 80 minutes when 5% had been hemolyzed; ●, after 150 minutes when 10% had been hemolyzed; △, after 240 minutes when 20% of the original number of cells had been hemolyzed.

hemolyzed the remaining 80% of intact cells showed fewer microcytes than at the earlier stages of the reaction.

As previously noted, controls of the defibrinated blood mixed with Tyrode's solution containing a dilution of sterile broth similar to that of the toxin, and incubated for the full period of the experiments showed no departure in form of the red cells from those in the freshly drawn blood.

Streptococcus and Tetanus Toxins.—Table 2 indicates the comparative rates of hemolysis in samples of the same 50% defibrinated blood by the last mentioned *B. welchii* toxin, a sample of *B. tetani* toxin and of streptococcus hemotoxin. The tetanus toxin was the fresh filtrate of a two-day culture of *B. tetani* (as used previously in experiments *in vivo*^{1, 4}) diluted 1:200. The streptococcus hemotoxin was the fresh filtrate of a two-day culture of an actively hemolytic erysipelas streptococcus diluted 1:40. These dilutions were selected in order to give a slightly more active hemolysis than produced by the *B. welchii* toxin. It may be observed from the table that the initial rates of hemolysis by both the tetanus and streptococcus toxins were somewhat greater than by the *B. welchii* toxin but that the streptococcus toxin reaction slowed down somewhat more abruptly and the tetanus toxin much more abruptly than the *B. welchii* toxin.

The conspicuous anisocytosis in the blood acted on by the *B. welchii* toxin, shown in figure 2, has just been commented on. In striking contrast to this condition films prepared at frequent intervals from the blood undergoing hemolysis by the tetanus and streptococcus toxins always exhibited normal red cells.

To confirm the opinion from visual comparison of films, size counts were made, as in the former cases, and the results with tetanus toxin plotted in figure 3. It is apparent from the graph that after 2, 4 and 5% of the cells had been hemolyzed those which remain show no measurable departure from the normal size. In figure 4 are results of counts of like sizes in the blood undergoing hemolysis by streptococcus hemotoxin. It appears that after 5, 10 and 20% of the cells had been hemolyzed those that remain intact show no definite variation from the normal size. Similar degrees of hemolysis by *B. welchii* toxin result at the same time in conspicuous anisocytosis in the unhemolyzed cells.

CONCLUSION

B. welchii toxin mixed with fresh defibrinated rabbit blood produces rapid destruction of erythrocytes, accompanied by a definite variation

of size. But in contrast with the action in vivo in which both microcytes and macrocytes are produced, the direct reaction in vitro results only in microcyte formation.

Tetanolysin and streptolysin in concentrations which produce similar amounts of hemolysis, when mixed with fresh defibrinated rabbit blood do not produce measurable variation in the size of the unhemolyzed cells.

This action of *B. welchii* toxin on red cells in the test tube seems comparable with the initial stages of the action of the toxin in vivo. In every case of welchii toxemia in rabbits whether following the injection of toxin or accompanying an infection which has been observed sufficiently early, there has been a primary development of microcytes which have persisted throughout the period of decreasing red cell numbers, but it has generally been observed that the minimum size of the red cells is reached and followed by some increase in size while the count is still falling.^{1, 4} This is similar to the in vitro observations which have just been described.

In the living animal, however, if it survives the primary anemia, during the period of stationary minimum numbers of red cells, or particularly during the period of increasing concentration of red cells in the blood, the microcytes are gradually replaced to a large extent by macrocytes, many of which exhibit polychromatic staining, poikilocytes and nucleated red-cells. This latter condition is evidently associated with erythrocyte regeneration and has not been observed in the test tube experiments.

⁴ Reed, G. B.; Orr, J. H., and Burleigh, C. H.: Canadian M. A. J., 1926, 16, p. 525.

THE GROWTH OF HEMOPHILIC BACILLI WITH CERTAIN IRON SALTS

JANET M. BOURN

From the Albert Kuppenheimer Fund of the Michael Reese Hospital and the Nelson Morris Memorial Institute for Medical Research, Chicago, and the Department of Hygiene and Bacteriology of the University of Chicago

It has been recognized that the so-called hemophilic bacilli, of which the Pfeiffer bacillus is a type, require the presence of two substances to permit growth in a nutrient medium. These have been designated as the X and V factors by Thjötta and Avery;¹ both may be found in blood and plant tissues. In their studies on bacterial nutrition Thjötta and Avery have adequately reviewed the literature concerning earlier recognition of the rôle of growth accessory substances. The work of these investigators has been essentially confirmed by Rivers and Poole² and by Kollath,³ who also have reviewed the literature with regard to growth requirements of the Pfeiffer bacillus.

The X factor is thermostable and has been associated chiefly with the blood pigment. For this reason it has also been thought of as iron-containing. This substance acts in minute quantities and has been looked upon as a biocatalytic agent and a peroxidase by Avery and Morgan.⁴

The V substance on the other hand is thermolabile and may be derived from watery extracts of yeast and of certain bacteria as well as from similar extracts of red blood cells and of various vegetables. This substance is active only in much higher concentration than is the X factor, and while its true nature has not been defined it appears to be vitamin-like. Work by Thjötta⁵ indicates that it is not identical with the water-soluble vitamin B, however.

Rivers⁶ has shown that some strains of the hemophilic bacilli which have been isolated require only the X factor or the V factor for growth, in contradistinction to those strains which demand the presence of both substances. This fact has been made use of by Webster and Baudisch,⁷ who in a study of the biology of *Bacterium leprosepticum* employed two types of inorganic iron compounds which they found to influence the growth of certain bacteria of the hemophilic, anaerobic, and hemorrhagic septicemic groups. One type of the inorganic iron compounds is represented by pentacyano iron salts and the other by certain oxides of which magnetite is an example. These iron compounds possess in common with hemoglobin the ability to absorb oxygen and to give a positive benzidine

Received for publication, June 28, 1927.

¹ J. Exper. Med., 1921, 33, p. 763; 34, pp. 97, 455.

² Bull. Johns Hopkins Hosp., 1921, 32, p. 202.

³ Centralbl. f. Bakteriöl., 1, O., 1924, 93, p. 506; 1925, 95, pp. 158, 279.

⁴ J. Exper. Med., 1924, 39, p. 289.

⁵ Ibid., 40, p. 671.

⁶ Bull. Johns Hopkins Hosp., 1921, 33, pp. 149, 429.

⁷ J. Exper. Med., 1925, 42, p. 473.

reaction. In addition, they show the so-called X type of biocatalytic activity. That is, they are able to support the growth of a hemophilic bacillus which requires only the presence of the X factor for growth in a broth medium, but cannot promote growth of those hemophilic bacilli which need only the V factor or both factors. This interesting study served as a basis for the initiation of the work reported in this article.

The following experiments were undertaken in the hope that the results would offer a method by which the metabolism of iron by certain strains of the hemophilic bacilli could be studied. For such a purpose it was desirable to obtain growth in a synthetic medium, the constituents of which would have a known chemical composition. Hemophilic bacilli which require only an X factor for growth seemed to be favorable for such an attempt.

Methods.—The strains of hemophilic bacilli employed in these experiments were isolated from the throats of different individuals and in one instance, from the spinal fluid of a patient with meningitis. They have been regarded as strains of the Pfeiffer bacillus, and have been maintained on slants of Levinthal agar⁸ which is essentially a coagulated whole blood medium. From such cultures, when not more than 24 hours old, inoculations have been made by means of a sterile platinum loop into the substrate to be examined for growth-producing qualities. The growth of the Pfeiffer bacillus on Levinthal agar is usually a very luxuriant one which in addition to offering a rich source of bacteria for the seeding of other mediums perhaps furnishes a smaller proportion of substances essential to the growth of this organism to be carried over into the menstruum under consideration. Most of the experiments have been carried out in a fluid medium on the basis that such a medium offers better conditions for the initiation of growth.

Transfers from fluid cultures to fluid mediums were always made by means of sterile pipets, whereas subcultures on Levinthal agar slants or plates which served to verify the presence of viable organisms were made by means of a sterile platinum loop.

The optimal hydrogen ion concentration of any medium for the growth of the Pfeiffer bacillus has been considered to be P_H 7.4 and such a hydrogen ion concentration has been employed throughout this study.

Pentacyano Iron Salts and Magnetite.—Of the pentacyano iron salts,⁹ sodium aquopentacyanoferroate, $(CN)_5Fe(H_2O)Na_3$ and sodium aminepentacyanoferroate * $(CN)_5Fe(NH_3)Na_3$ have been tested with respect to their influence on the growth of some hemophilic bacilli.

⁸ Levinthal, N.; Kuczynski, M. H., and Wolff, E.: Die Grippe Pandemie von 1918, 1921.

⁹ Hofman, K. A.: Ann. d. Chem., 1900, 312, p. 1.

* Through the generosity of Dr. Webster and Dr. Baudisch some of the above pentacyano salts which had been prepared by Dr. Baudisch, were available for the earliest pentacyano experiments carried out. Subsequent experiments were made, using sodium aquo-ferroate prepared by myself according to Hofman's formula.⁹

One cc. of a 0.2% sterile aqueous solution of these salts was the amount usually added to 5 cc. of the medium under consideration. Magnetite, Fe_3O_4 , (Merck's magnetic iron oxide) was tested for similar activity and was employed in amounts of at least 0.5 gm. to 5 cc. of fluid medium. Magnetite proved to be much less desirable to use, as it reacted with the medium in which it was introduced causing an increased alkalinity. In a broth medium this change in reaction occurred more slowly and was less marked than that which was observed in the synthetic medium used in this work. For example, synthetic solutions, which are described elsewhere in this paper, showed a hydrogen ion concentration of P_H 8.1 after having been incubated overnight at 37 C in the presence of magnetite. The hydrogen ion concentration had previously been P_H 7.4. Veal infusion broth, on the other hand, when treated similarly with magnetite changed from P_H 7.4 to P_H 7.6. After standing in contact with magnetite for more prolonged periods a greater alkalinity in broth was noted. Distilled water which was allowed to remain in contact with the magnetite did not show an alkaline reaction to phenol red. As a result of this reaction in mediums, magnetite has been tested only in broth cultures.

TABLE 1.
INFLUENCE OF PENTACYANO IRON SALTS AND MAGNETITE UPON THE GROWTH OF
HEMOPHILIC BACILLI IN VEAL INFUSION BROTH

Mediums	Subcultures of Serial Transfers	Strains			
		1	2	3	4
Broth + Sodium aquopenta- cyanoferroate *.....	1	++	++	++	++
	2	++	+±	0	0
	3	++	+±	0	0
	4	++	—	—	—
	5	++	—	—	—
Broth + Magnetite.....	1	++	++	+±	+
	2	++	0	0	0
	3	0	0	0	0
Plain broth.....	1	++	+±	+±	+±
	2,3	0	0	0	0

* Sodiumamine-pentacyanoferroate acted in a similar manner.

In this and the succeeding tables the meaning of the symbols used is as follows: ++, good growth; +±, moderate growth; +, numerous colonies; ±, slight growth; 0, negative results; —, not tested.

Table 1 illustrates the activity of the pentacyanoferroate and magnetite in stimulating the growth of certain strains of hemophilic bacilli in a veal infusion broth medium. It is seen that pentacyanoferroate can support the growth of a strain of hemophilic bacillus which requires only the X factor in a broth medium through a series of transfer cultures. Strains 1 and 2 appear to be of the X type while strains 3 and 4 seem to require more than the X factor for continued growth in broth. The growth of hemophilic bacilli in plain veal infusion broth cannot be demonstrated beyond the primary culture in the series, although viable organisms can be shown to be present for longer or shorter periods in this medium.

With the magnetite used it has only been possible to demonstrate growth of a hemophilic bacillus of the X type through two serial trans-

fers. Its biocatalytic activity appears to be definite, but much more feeble as compared with the pentacyano ferroate. These results substantiate the report of Webster and Baudisch with respect to the biocatalytic activity of these iron compounds. It is interesting to remark that not only has the growth of the X type of hemophilic bacilli been observed through five generations in the instance of strain 1, but that viable organisms have been shown to be present in these cultures after they have been maintained from 20 to 30 days at room temperature.

When the pentacyano iron salt solution was added to veal infusion agar and plates of such agar were seeded with hemophilic bacilli of the X type only a slight growth resulted. This growth when transferred to a second iron salt agar plate produced a few well defined colonies of typical bacilli. The firm medium appeared to be less suitable to induce growth.

Synthetic Mediums.—Two different synthetic solutions have been used in this study. One, which is fairly simple, has been described by Nelson¹⁰ and corresponds to the following formula:

Distilled water	1000.0 cc.
Diabasic potassium phosphate.....	2.0 gm.
Ammonium succinate	2.0 gm.
Sodium chloride	2.0 gm.
Dextrose	2.0 gm.

The other represents a slight modification of the nonprotein synthetic medium employed by Long and Seibert¹¹ for the production of tuberculin. The following formula was used in the experiments reported.

Asparagine	5.0 gm.	Sodium chloride	2.0 gm.
Ammonium citrate	5.0 gm.	Magnesium sulphate.....	1.0 gm.
Potassium acid phosphate.....	3.0 gm.	Glycerol	50.0 gm.
Sodium carbonate (anhydrous). 3.0 gm.		Water	1000.0 cc.

The above combination was altered from the original, only in the omission of a trace of ferric ammonium citrate and, on occasion, the glycerol; the latter, at times, was added in a concentration of less than 5%. Dextrose has also been used to replace as well as to supplement the glycerol. This medium was selected for use in preference to the simpler synthetic medium of Nelson, since the addition of the pentacyano iron salt does not result in an iron precipitate as occurs when the salt is added to the Nelson medium or veal infusion broth. The ability of the asparagine medium to hold the iron in solution may be ascribed to the presence of the citrate.

¹⁰ J. Infect. Dis., 1926, 38, p. 371.

¹¹ Am. Rev. Tuberc., 1926, 13, p. 393.

Table 2 illustrates the ability of four different strains of hemophilic bacilli to grow in asparagine synthetic medium containing sodium aquopentacyanoferroate as compared to similar medium to which yeast extract had also been added. Strains 3 and 4 appear to require the presence of both X and V factors, whereas strains 1 and 2 give evidence of growth when only the X factor in the form of pentacyano iron salt is present. It is observed that viable organisms could not be demonstrated in any synthetic culture at the end of 72 hours of incubation at 37 C. It is also to be noted that although viable organisms could be revealed by subculturing on Levinthal agar, subcultures in synthetic solutions made at the same time failed to show demonstrable growth. It was thought, however, that if transfers were made to synthetic solu-

TABLE 2
GROWTH OF HEMOPHILIC BACILLI IN SYNTHETIC MEDIUM AS SHOWN BY
SUBCULTURES ON LEVINTHAL AGAR

Strain	Period (hours) of Incubation Before Subculture	Growth of Subcultures on Levinthal Agar from Asparagine Synthetic Medium, pH 7.4			
		Plus Na aquo- pentacyano- ferroate	Plus Na aquo- pentacyano- ferroate and yeast extract	Plus Na aquopenta- cyanoferroate and sterile emulsion of strain 1.	(Without additional substance)
1	20	± (delayed)	++	± (delayed)	—
	24	++	—	—	+
	48	++	++	0	0
2	48	±	++	—	0
3	48	0	+	—	0
4	48	0	++	—	0

Subcultures made after 72 hours of incubation were all negative.

tions from the original synthetic cultures after only 24 hours of incubation growth might be demonstrated in the synthetic subcultures. Experiments to illuminate this point were carried out using strain 1 which was selected as a hemophilic strain requiring only the X factor for growth. The results of these experiments are typified by those under strain 1 in table 2.

Viable organisms were present in the iron salt culture at the end of 48 hours of incubation, but at the end of 72 hours no growth could be obtained on a Levinthal agar subculture. No growth could be demonstrated in secondary iron salt synthetic cultures made either at the end of the 24 hour or 48 hour period of incubation. Considering the control culture of plain synthetic solution, it is apparent that viable organisms can be revealed at the end of 24 hours, but not at a later period. Occasionally very slight growth occurred on subculture of a primary cul-

ture in plain synthetic medium at the end of the 48 hour period of incubation. The addition of some sterile emulsion of the homologous hemophilic bacillus to synthetic medium did not particularly enhance the growth of the organism (strain 1).

Subcultures were made from the supernatant culture fluid and also from the mucoid bacterial sediment. The latter gave rise to more luxuriant subcultures on Levinthal agar in certain instances, perhaps due to a matter of heavier seeding. The seeding of synthetic mediums with portions of such mucoid sediment was not successful with respect to inducing growth in secondary synthetic cultures.

Attention should be called to the character of the growth of strains 1 and 2 in the synthetic medium. Very little turbidity of the solutions occurred; the growth appeared principally as a mucoid sediment arising from the loopful of bacteria which had served as an inoculum. This mucoid bacterial mass which forms during incubation is considerably greater than the original inoculum and when the culture fluid is agitated it rises up in a thread-like whirl. A similar type of growth has been observed at times in broth cultures of these strains, and indeed, the luxuriant confluent growth which occurs on moist Levinthal agar has a mucoid character. It is interesting to remark that these strains throughout their period of observation have not shown pleomorphic changes to any marked degree, but have maintained the appearance of very small coccoid bacilli typical of the Pfeiffer bacillus.

An experiment was then performed in which organisms of the X type (strain 1) were subjected to three washings in plain synthetic solutions before being inoculated into synthetic mediums. As a control some of the unwashed organisms were inoculated into corresponding mediums. Good growth was obtained in subcultures on Levinthal agar after 22 hours of incubation in the instance of the primary cultures of unwashed organisms, whereas the subcultures made from the cultures of washed bacilli remained sterile. A Levinthal agar slant inoculated with some of the washed bacilli at the time that the synthetic mediums were seeded showed a luxuriant growth after overnight incubation. Subcultures made at the end of 48 hours showed no growth except a very slight one in that of the unwashed bacilli in plain synthetic medium. All subsequent subcultures, synthetic as well as Levinthal medium, remained sterile.

These results would indicate that the slight growth which occurs in the primary synthetic cultures is due to growth accessory substances introduced in the unwashed inoculums taken from whole blood agar.

As a matter of control four other salts have been tested for the possible oligodynamic action which they might exert on the growth of certain strains of hemophilic bacilli. Two of these salts contained iron, one having the ferric ion and representing an organic salt, namely ferric ammonium citrate, and the other being an inorganic salt with the ferrous ion present, or ferrous sulphate. The remaining two salts, one organic and the other inorganic, were zinc acetate and zinc sulphate. These salts were added in a concentration of 1:20,000 to asparagine synthetic solution which contained only 1% glycerol and 0.5% dextrose.

Only subcultures on Levinthal agar were made at the end of 12 and 20 hours of incubation. The 12 hour subcultures showed no growth. Of the 20 hour subcultures only growth of strain 1 was obtained in the instances of the ferrous sulphate and zinc acetate cultures.

A somewhat similar experiment was performed in which ferric ammonium citrate, ferrous sulphate, and zinc acetate were the only salts tested. On this occasion the salts were added in concentrations of 1:10,000 and 1:20,000 to asparagine synthetic solution which contained no glycerol or dextrose and in a concentration of 1:20,000 to synthetic solution containing 0.5% dextrose. All of these portions were combined with 2% agar and plated. The various plates were divided into sections and seeded with three strains of hemophilic bacilli and a strain of *Bacterium coli*. No growth occurred except in the cultures of *Bact. coli*.

DISCUSSION

With the knowledge in mind that certain inorganic iron compounds have been found which appear to function as the X factor in relation to the growth of some hemophilic bacilli,⁷ an attempt was made to secure growth of such organisms in a chemically defined synthetic medium. In such a medium, in which the only organic constituents were asparagine, ammonium citrate and glycerol, the X factor was represented by sodium aquopentacyanoferroate. When this medium was inoculated with hemophilic bacilli which required only the X type of biocatalytic activity in a broth medium, viable organisms could be demonstrated after 48 hours of incubation at 37 C, but not at a later period. In synthetic medium which contained no pentacyanoferroate viable organisms could be revealed after 24 hours, but usually not at a later period of incubation. No success attended attempts to secure growth in secondary synthetic cultures. Synthetic medium to which either yeast extract or sterile emulsion of the homologous organism in salt solution had been added in addition to the iron salt did not yield better results.

When washed bacilli were used to seed the synthetic medium, no viable organisms could be demonstrated by subcultures made at any period of incubation. That pentacyano iron salts play a very important, though not an independent rôle, in the stimulation of growth of certain hemophilic bacilli is indicated by the different manner of growth which occurs in broth and in synthetic medium to which pentacyanoferroate has been added. In the iron salt broth, good growth is secured in numerous serial transfers and the cultures have been found to be viable after having been maintained at room temperature for one month. The inoculation of similar hemophilic bacilli into plain veal infusion broth results in moderate growth in the primary culture. This growth persists for a longer or shorter period but cannot serve to initiate growth in a second plain broth culture.

Comparing the type of growth obtained in the primary cultures of synthetic medium with that occurring in plain broth, it would seem that they are essentially analagous. It is evident that the initial growth is due to the growth accessory substances introduced with the bacterial inoculum taken from whole blood agar. These accessory substances become exhausted or so diluted that their activity to stimulate growth is lost in serial transfers.

Salts other than the pentacyano iron compounds were tested for the oligodynamic action which they might exert on the growth of these hemophilic bacilli. In experiments in which ferrous sulphate, ferric ammonium citrate, zinc sulphate, and zinc acetate were employed, only sporadic growth was encountered in the instance of ferrous sulphate and zinc acetate. Magnetite when present in broth cultures gave some evidence of biocatalytic activity of the X type.

It is interesting to recall that Thjötta and Avery¹ (1921) have reported that Uschinsky's nonprotein synthetic medium plus sterile raw potato suffices to support growth of *Bact. influenzae*. They also found that potato in plain buffer solutions of Na and K phosphate (M/15, P_H 7.5) supported growth. They conclude, "It is evident then that potato contains both the V and X substances and that these factors together with the native protein and carbohydrate of potato can replace in media blood pigment and tissue derivatives from animal sources."

It is noteworthy also to observe in this connection the recent work of Kollath¹² in Germany concerning the influence of irradiation on the mineral metabolism of the influenza bacillus. Kollath preferred to use

¹² *Centralbl. f. Bakteriol.*, 1, O., 1926, 100, p. 97.

a firm medium, a meat infusion agar, to which no peptone had been added. In all of his experiments the influenza bacillus was grown in symbiosis with other bacteria, a coccus form and a strain of Friedländer's bacillus. Such growth is denoted in the German literature as "Ammenswachstum" or "wet-nurse" growth. Under such methods, he tested potassium ferrocyanide and manganous chloride, both before and after irradiation. By the addition of nonirradiated potassium ferrocyanide to agar no "wet-nurse" growth of the influenza bacillus occurred. However, when this iron salt was irradiated, a "wet-nurse" growth of the Neisser type resulted, depending upon the duration of the irradiation. The same results could be attained by the use of irradiated manganous chloride, which would indicate that manganese can replace iron to a certain degree. The irradiated iron salt, Kollath found, could at no time produce growth of the influenza bacillus by itself; the activity of living plant cells was always essential. He concludes, therefore, that the X factor cannot be gained from mineral salts without bacterial activity. At the same time, Kollath observed that through irradiation the iron salt gained the ability to give a positive benzidine reaction similar to the peroxydase reaction of the blood, also that the catalase reaction was increased or appeared for the first time.

Kollath deduces from this evidence that an increased oxygen activity of the iron salt occurred under the influence of light. Since he could not demonstrate the formation of an "inorganic vitamin" by irradiation of iron, Kollath extended his experiment with the purpose of gaining a more exact knowledge of the growth-accessory substance designated as the V factor and expresses the thought that only out of a combination of V substance and X substance can vitamin action with respect to bacteria arise. He has attempted to explain the chemical nature of the substance V on the basis of the more recent vitamin investigations and bases his own experiments on this work. He finds an analogy in the water-soluble phosphatides of Cranmer¹³ to the watery extract of the V substance which contains phosphatides. Under the influence of light phosphatide can be split into highly active aldehydes which can activate the minerals. When these activated minerals are transported to the living plant cell the X factor can act. In conclusion, he theorizes, "The united action of phosphatide and mineral produces subsequently that which we are accustomed to consider as vitamin-action concerning the bacteria."

¹³ Meldinger fra Norges Landbrukshøiskole, 1922, 2. Cited by Kollath.

In the experiments of Kollath,¹² and of Thjötta and Avery,¹ as well as of other investigators, the growth-stimulating properties of the X and V substances have always been demonstrated in the presence of fresh plant tissue or tissue derivatives either from animal or plant sources. For this reason the true independent action of either factor has been masked. The fact that continued growth of certain hemophilic bacilli can be secured in a veal infusion broth to which pentacyano iron salt has been added and not in a synthetic medium containing the iron salt, or in plain veal infusion broth, indicates that the biocatalytic activity observed must result from an interaction of the pentacyano iron salt and some tissue derivative of the veal infusion base of the broth medium. It is assumed by comparison of the benzidine reactions that the iron salt is as active in the synthetic medium as in the broth medium.

Since this paper was accepted for publication another pertinent article, by Valentine and Rivers,¹³ has appeared. These investigators, in making further observations concerning growth requirements of hemophilic bacilli, obtained results which appear to "indicate that strains of hemophilic bacilli requiring the addition of only V or X to their media are capable of supplying or acting as X or V respectively to an extent sufficient for the needs of other bacilli that require the addition of these factors to their media." This occurrence of true symbiosis would seem to confirm further the deductions drawn from the evidence presented in this paper as well as from that of other workers.

SUMMARY

Certain hemophilic bacilli can be cultivated through successive transfers in veal infusion broth to which sodium aquopentacyanoferroate has been added, whereas, homologous bacilli do not survive beyond the primary culture when the iron salt is not present.

In a chemically defined synthetic medium which contains the iron salt, these hemophilic bacilli give evidence of growth in the primary culture when an unwashed bacterial inoculum has been used, but this does not serve to initiate growth in a secondary synthetic culture.

The evidence suggests that pentacyano iron salts do not function independently as the X factor in bacterial nutrition, but that the biocatalytic activity observed is the result of an interaction between the inorganic iron salt and some thermostable tissue derivative from an animal source.

¹³ J. Exper. Med., 1927, 45, p. 993.

THE ETIOLOGY OF MEASLES

RUDOLPH DEGWITZ

From the University of Greifswald, Germany

The first scientific experiments on measles were done in England in the 18th Century. In Europe that century was looked on from a medical point of view as the century of variolation, few discoveries attracting such wide public interest as variolation against smallpox. Even Jenner's vaccination did not arouse so much interest as the older method.¹ With the beginning of the 18th Century smallpox raged in Europe, and a new method of immunizing against this disease came from the East. Smallpox virus as used at that time was planted in the skin of susceptible persons. After such an inoculation of the skin the person did not develop typical smallpox, which showed at that time a mortality rate of 30% or more, but developed a disease which began sooner after the inoculation than the natural disease, was shorter and much milder, but gave, nevertheless, a lasting immunity. It was natural then, considering the similarity of the clinical pictures of smallpox and measles, that the same method should be applied to measles.

Home² who tried this method or at least was the first to publish it, stated: "Considering how destructive this disease is in some seasons; considering how many die, even with the mildest constitutional form; considering how it hurts the lungs and eyes, I thought I should do no small service to mankind if I could render this disease more mild and safe in the same way that the Turks have taught us to mitigate smallpox. I suspected strongly that the cough, often so harassing, even of the mildest kind, was produced by receiving the infection mostly by the lungs and I hoped that the symptom would abate considerably if I could find a method of communicating the infection by the skin alone." Home tried to use the blood of measles patients and skin scales following the appearance of the rash. He reported measles following skin inoculations which was analogous to smallpox after variolation. The symptoms began earlier than in the classical disease and were much milder and of shorter duration.

Received for publication, July 13, 1927.

¹ Klebs: *Die Variolation im 18 Jahrhundert*, 1914.

² *Medical Facts and Experiments*, 1759, 8.

In several countries up to 1850, efforts had been made to repeat Home's experiments but no one was able to confirm his report, so the professional critic finally made up his mind that Home's children living in the same house with measles patients had not been infected by Home's skin infection, but in the natural way, and had developed by one chance or another a very mild form of measles. The idea that the organisms causing measles could, by entering the human system through the skin and not by their natural entrance—the mucous membranes of the respiratory tract—produce a much shorter and milder disease, was dropped. It remained uncertain whether the blood of measles patients contained the measles organism.

Hektoen³ proved beyond doubt that the primary cause of measles is contained in the blood of measles patients soon after the appearance of the rash, and that blood drawn at this time and injected into susceptible human beings would produce measles. Hektoen withdrew the blood once immediately after the appearance of the rash and once 30 hours later, and diluted it with 3 to 12 parts of ascitic fluid. He placed this mixture in the incubator for 24 hours and injected 4 to 5 cc. subcutaneously. These experiments were repeated and the results confirmed by Degkwitz. It was found that on some occasions the organisms causing measles had disappeared from the blood 24 to 30 hours after the appearance of the rash, and that 12 to 24 hours before the appearance of the rash the blood was in some cases not yet infectious, but every time the blood was drawn at the time the rash first appeared on the face, the attempt was successful.

Experiments were also made to determine how to preserve the organisms causing measles. Hektoen's idea of diluting the infectious blood was followed, but ascitic fluid did not seem to be the most suitable medium for preserving its infectivity. The common solid culture mediums and plain broth and modified broths were found not to be suitable. Starting with the idea of keeping the organisms causing measles in surroundings as nearly natural as possible, the blood was diluted with salt solution containing the same number of anions and cations and showing the same P_H as the blood.* It was found that infectious blood diluted with such a physiologic salt solution in the proportion of 1:7 to 1:10 maintained its infectivity for several weeks when kept at 0 C.; 0.5 to 1 cc. of such a mixture injected subcu-

³ J. Infect. Dis., 1905, 2, p. 283.

* "Normosal," a sterile, dry, buffered salt in ampules, was used throughout these experiments.

taneously or intramuscularly produced typical measles at the normal time in susceptible persons.

These facts having been established, Home's experiments could be transferred to institutions and towns where there were no measles cases at the time of the experiment, and it could be determined whether an inoculation into the skin with this material produced a kind of measles different from that produced by inoculation on the mucous membrane of the respiratory tract. Certain susceptible persons were given intradermal injections of 0.2 to 0.5 cc. of a 1:7 dilution of infectious blood, while others received the same amount in the nasopharynx. The persons inoculated in the skin had measles: Koplik's spots and a sharp rise of temperature on the 9th day, a rash on the 10th day, and an abrupt return to a normal temperature on the 11th day after inoculation. In this type of measles, from intracutaneous inoculation, the development was sooner than in either classical measles or the measles produced by nasopharyngeal inoculation, the course was milder and shorter than after the nasopharyngeal inoculation, and the mucous membranes of the respiratory tract were not visibly affected.

These experiments indicated that there seemed to be an analogy between morbillisation against measles and variolation against smallpox. It has been noted that the organism causing measles, when inoculated in the skin, behaved in somewhat the same way as smallpox virus similarly injected. Hektoen reported that the blood causing measles was sterile from a bacteriological point of view. It could be proved that infectious blood originally sterile from a bacteriological point of view was capable of producing measles. It could be proved, furthermore, that sterile Berkefeld filtrates of infectious blood, are capable of producing measles. The sterility of the original blood and of the filtrates was proved by planting the material in different mediums, both aerobic and anaerobic. As a filtrable virus may be defined as an organism passing a filter candle which removes bacteria, these experiments showed that the cause of measles is a filtrable virus.

Culturing the Virus.—Infectious blood diluted with physiologic salt solution as described above kept its infectivity in the incubator as long as seven to eight days. As a rule in these experiments, when the whole blood was incubated, the red corpuscles were thrown out by centrifugation, or a clear plasma dilution was taken off after the corpuscles had settled. The plasma dilution after removal of the red corpuscles by centrifugation proved to be infectious as well as the part containing

the red corpuscles. If such a dilution of infectious plasma after incubation for 7 or 8 days proved to be infectious, had the virus merely been kept alive during this time or had it grown? The microscope, at least so far as it was used, was of no use in deciding this question which had, therefore, to be decided in another way.

If the minimum infectious dose were roughly determined before the material was placed in the incubator and the infectious material carried through several generations, by subculturing in the same way as bacteria are cultured, a dilution of the original material could be reached far beyond this minimum infectious dose. When such a dilution should reach 10,000 or 100,000 times as high as the dose of original infectious material which failed to infect, and should prove in these dilutions to be able to produce in susceptible persons measles or a febrile reaction followed by immunity, the question whether the virus had grown or had only been diluted would be answered.

For the choice of a culture medium for the virus, the original idea to put the virus under conditions as nearly natural as possible was maintained. It was planned to put the virus into plasma from a person susceptible to measles and to dilute this plasma with a buffered salt solution which was as much like blood as possible. It was planned to associate the measles virus with living cells, the cornea or lens cells of the human fetus being used originally for this purpose. Later, the virus was associated with slowly growing bacteria, such as pneumococci and streptococci, which are found regularly during an attack of measles and which seemed to be in a natural way associated with the measles virus. It was, however, soon found that the growth in vitro of those organisms associated with the virus had to be kept down in numbers. If these bacteria were allowed to grow at will they overgrew the virus and killed it.

To prepare a culture medium for measles virus two things were necessary: first, plasma of persons susceptible to measles (adult blood may be used without reference to susceptibility to measles), and second, a bacterial suspension of known concentration to be grown in symbiosis with the measles virus. The human plasma can be obtained in two ways: sterile bleedings can be made with paraffined needles and the drawn blood kept and centrifugated in paraffined tubes, or the sterile blood may be mixed with a sodium citrate solution so that the amount of citrate in the whole blood is not higher than 5 to 6 parts per thousand. After centrifugation at a low speed the plasma is kept on ice.

A known concentration of bacteria to be used as symbiotic organisms is obtained in the following way: several species of the streptococci (Tunncliffe's organisms as well as others) are grown over night in common broth and this growth diluted 1:50 or 1:100, with salt solution. One cc. of human or sheep plasma is now diluted with 6 to 7 cc. of physiologic salt solution, several flasks being prepared in this way. The bacterial suspension is tested by placing 1 drop in the first of 10 flasks containing the mixture of plasma and physiologic salt solution; into the second flask two drops, and so on up to ten drops. If the plasma was obtained by bleeding with paraffined needles into paraffined tubes, the plasma will clot when transferred and mixed with salt solution. If the blood is used as a sodium citrate mixture, the calcium precipitated by the sodium citrate must be replaced. In order to calculate the amount of sodium citrate contained in the plasma it is assumed that the whole amount which was added to the blood is contained in the plasma which has been carefully separated. The volume of plasma is accurately determined and the amount of sodium citrate contained in 1 cc. is calculated, one-half this amount of calcium chloride being required to cause the plasma to clot. For convenience sterile 20% solution of sodium citrate and 10% solution of calcium chloride are kept at hand.

The mixtures of plasma, physiologic salt solution, and bacterial suspension are incubated five to six days. The bacterial growths in this semisolid medium, after five or six days, should be seen with the naked eye. If, for example, 7 or 8 drops of the bacterial suspension show a visible growth after five or six days, the right amount of bacterial suspension added to the future growth of measles virus should be about one to two drops.

The culture for growing the measles virus is now made in the following way: One cc. of human plasma is placed in a small Erlenmeyer flask. To this is added 1 cc. of the dilution of originally infectious blood in physiologic salt solution. There are next added one to two drops of the bacterial suspension and the mixture is diluted with 6 to 7 cc. of the physiologic salt solution mentioned above. If the human plasma was obtained by bleeding in paraffined tubes the mixture will clot spontaneously, but if the plasma contains sodium citrate the amount of calcium chloride is added as indicated above. Owing to the possibility of contamination it is advisable to make several duplicates of each culture. The flasks are closed with rubber stoppers to prevent evaporation and incubated 8 to 9 days.

After this time the clot is broken by shaking and 0.5 to 1 cc. of this mixture is planted into a new mixture of plasma and physiologic salt solution. With the 0.5 or 1 cc. of the first generation enough symbiotic organisms are carried into the second generation. The second generation is incubated 4 to 5 days and then a mixture in the same way is carried into the third generation, the fourth, and so on. The amount of bacterial growth must be watched very carefully and the mixture passed through Berkefeld filters as soon as the colonies can be seen with the naked eye. When this is done, the sterile filtrate is planted into a new plasma mixture and a new amount of symbiotic organisms is added. It is better to filter as seldom as possible because filtering brings in another factor little known and therefore very difficult to control. After the second generation, sheep plasma may be gradually substituted for human plasma adding 25% at each transfer until at the sixth generation sheep plasma alone is used.

If infectious blood is not available the culture can be started from nasal secretions and sputum. The best time to collect this material is the preeruptive stage of the disease. The patients are asked to expectorate, after coughing, into a mixture of 1 part of human plasma and 20 parts of physiologic salt solution. About 0.5 cc. of sputum from older children or adults is sufficient for this inoculation. With younger children the material is obtained by placing several cc. of the salt solution in the mouth and inducing the child to eject the material into the mixture of plasma and salt solution. In either case the sputum-plasma mixture is incubated from 12 to 24 hours, the length of time depending on the amount of bacterial growth since one has always to fear that the virus will be killed by overgrowth of the bacteria. After incubation the mixture is diluted with physiologic salt solution in the proportion of 1:1, passed through a Berkefeld filter and 1 cc. of the filtered mixture cultured in the same way as the diluted blood, introducing symbiotic organisms. If the infectious material is obtained early in the disease, at the time when the sputum is poor in bacteria, the virus can be carried through generations in the same way as bacteria are cultured. But this method is not so safe as the one described above because of the presence of an uncontrollable number of bacteria.

Virus cultures have been carried 16 to 18 generations and proved capable of causing specific reactions when given subcutaneously to susceptible human beings. These specific reactions are only in the minority of instances typical measles. As a rule the infected individuals show

between the 10th and the 14th day after the injection a rise of temperature, conjunctivitis, rhinitis, bronchitis and stomatitis. In some cases there appears a rash which is, however, never a classical measles rash but which shows the bluish red occasionally seen in toxic measles cases. Prostration and subnormal temperature have been seen, while at other times the individuals seemed scarcely to be sick. In these cases no rash was seen so it had to be proved that the infection was a specific one, and it also had to be proved that the reaction was not due to the growth of the symbiotic organisms. Therefore, in most instances the symbiotic organisms were cultured alone, not associated with the virus, but grown in the same culture mediums. Injections of bacteriologically sterile filtrates of these organisms grown in this way caused no reactions. In the following manner it was proved that the reactions following an injection of cultured virus were specific: after a varying time they were reinjected with infectious blood, the infectivity of which was proved in untreated persons. It was shown that the persons who had reactions after the injection of cultured virus were later immune against high doses of infectious blood, while persons who received sterile filtrates of the symbiotic organisms alone, were later susceptible to a small dose of the infectious blood.

Experimental Measles in Monkeys.—In the following pages two experiments performed recently are described. Two strains of virus were used. Strain 1 was collected from a soldier on the third day of his rash. It was too late to obtain the blood virus so the soldier expectorated into a dilution of 1 cc. of human plasma in 20 cc. physiologic salt solution. This mixture was carried away and after 12 hours was placed in the incubator and left over night. It was then diluted 1:1 and filtered. One cc. of the sterile filtrate was inoculated in 1 cc. of human blood plasma diluted 1:7 as described above and incubated for 8 days, then subcultured as before. The material was then filtered and placed with symbiotic organisms and incubated four days. It was then diluted 1:1, filtered, and 1 cc. of the filtrate after it had been proved to be bacteriologically sterile injected subcutaneously into two monkeys (1 and 2). During the febrile reaction monkeys 1 and 2 were bled and their mixed blood inoculated into two other monkeys (4 and 5). Transfers were made in turn to monkeys 8 and 9, and from these to monkeys 10 and 11, pooling the blood for each transfer. Monkeys 3, 6 and 7 were used as controls—monkey 3 receiving uninoculated culture medium, and 6 and 7 no treatment though they were kept under the same conditions as the

inoculated animals. Charts of the temperature of these monkeys, exclusive of chart 7 which is essentially like chart 6, are shown in figure 1.

The monkey (*Macacus rhesus*) must be regarded as an unsatisfactory animal for experimental work on measles. From the experiences of other scientists, and my own with about 80 monkeys, it can be said that there is a high percentage of these animals not reacting at all to large amounts of undoubtedly infectious material.

One cc. of a mixture of 1 part of infectious blood in 7 parts of physiologic salt solution caused typical measles in a human being with the appearance of Koplik's spots and a rise of temperature on the 10th day, and a rash on the 12th day after inoculation, while 20 to 40 cc. of the same mixture injected into each of four monkeys failed to cause a satisfactory reaction in any of them. Typical measles, so typical that another observer might hold that the monkey had come down with measles, has never been seen by myself. Both the virus out of the incubator and the virus carried along from monkey to monkey seemed to be very virulent from the mortality rate of the animals (5 deaths in 8 monkeys). Necropsies were performed and in only one animal was definite reason for the death found, the cause of death in monkey 9 being pneumonia. None of the controls died or showed at the time any sign of illness.

A definite way to prove that a reaction in a monkey has been a specific one is to show that the monkey serum after the reaction will protect infected human beings. Normal monkey serum will not do this. Therefore, it was planned to prove that the monkeys referred to above had measles by using their convalescent serum as a preventive measles serum.

Experimental Measles in Human Beings.—Strain 2 of measles virus was collected from a child, bled just at the beginning of the rash. This blood was diluted in the proportion of 1:7 in physiologic salt solution, placed on ice and carried two days by train. One cc. of the mixture was injected into a person who came down with typical measles * with the appearance of Koplik's spots, rise of temperature, and rash on the 10th to the 12th day after inoculation. One cc. of the same material diluted 1:5000 was injected at the same time into another person who showed no reaction. Both of these subjects had been under continuous medical observation since birth and neither had ever had measles.

* This case was in the incubation stage of varicella when inoculated, the eruption of varicella appearing on the day following inoculation.



Fig. 1.—Temperature charts showing reaction of monkeys to virus cultures and to blood of monkeys thus infected.

To determine whether measles virus could be grown in culture mediums as described above, it was assumed that it could be regarded as a proof of growth if the same material used in those two cases and proved to be unable to infect in a dilution of 1:5000 could be carried for several generations until the dilutions went far beyond 1:5000. The experiment was planned so that strain 2, unable to infect in a dilution of 1:5000 of the original infectious blood, would be carried at least to a dilution of 1:5,000,000 by subculturing, and strain 1, obtained earlier, would be in much higher dilution.

After diluting by culturing, and incubating, to allow growth to occur, it was planned to make injections in persons susceptible to measles and to watch their reactions. A control injection made at the same time in another susceptible person, with a sterile filtrate of symbiotic organisms (typical and atypical Tunncliff strains) which had been carried alone in the same culture mediums and in the same manner as the virus. Whatever might follow these injections with strain 1 or 2, and with a sterile filtrate of the symbiotic organism alone in the control, both groups were to be given second injections with undoubtedly infectious material, such as blood drawn just at the beginning of the rash. The temperature charts showing the reactions following the first injection of these three persons are shown in figure 2.

A person designated as A received subcutaneously 1 cc. of filtered, bacteriologically sterile culture of strain 1 carried into the 15th generation which reached a dilution of the originally infectious material of 1 in 3 trillions. The material had been 78 days in the incubator.

Person B received 1 cc. of the filtered, bacteriologically sterile culture of strain 2 carried into the 8th generation, incubated for a total of 45 days, and containing the originally infectious material in a dilution of 1 in 40 millions.

Person C received 1 cc. of a sterile filtrate of symbiotic organisms alone grown on the same culture mediums and cultured in the same way as the virus.

Two months later the three persons A, B and C were reinoculated and at the same time an experiment performed to show that the serum of the monkeys demonstrated above was able to protect undoubtedly infected individuals against the disease. C, treated two months before with a sterile filtrate of the symbiotic organisms alone, received an injection of 2 cc. of a dilution of 10 cc. of infectious blood in 100 cc. of physiologic salt solution and came down with typical measles, as the temperature chart shows (fig. 2). A, treated two months before with

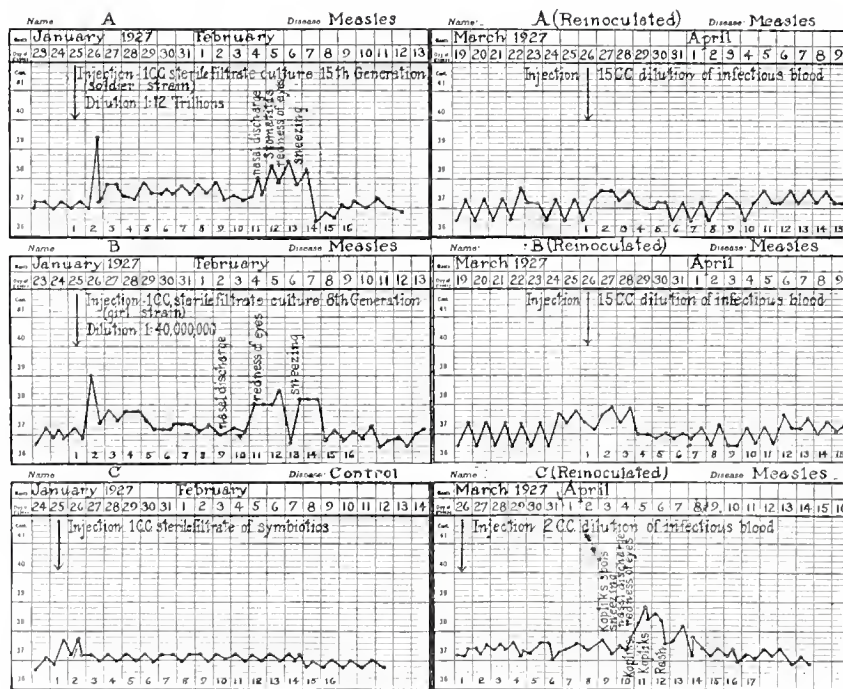


Fig. 2.—Temperature charts showing reactions to sterile filtrates of virus cultures in A and B with protection against reinoculation; and in C, lack of reaction to filtrate from symbiotic organisms alone and no protection against reinoculation.

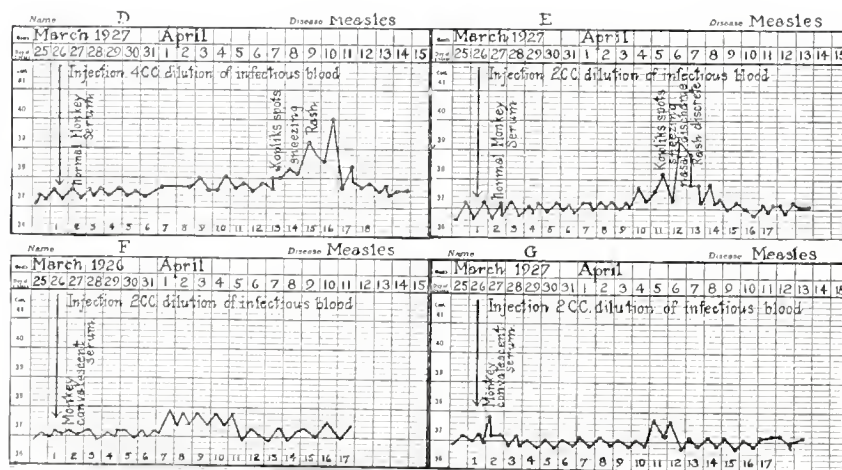


Fig. 3.—Temperature charts for persons given infectious measles blood with an injection of monkey serum 24 hours later. D and E who received normal monkey serum were not protected; F and G who received serum from monkey convalescing after treatment with virus, were protected.

the 15th generation of strain 1 diluted to 1 in 3 trillions, and B, treated two months before with the 8th generation of strain 2, diluted 1 in 40 millions, received an injection with 15 cc., an amount $7\frac{1}{2}$ times larger than the amount given to C. Both A and B proved to be immune against this large amount of infectious blood (fig. 2).

A person D received an injection of 4 cc. of the same infectious blood and salt solution; and E received 2 cc. of the same mixture. Each received, 24 hours after this injection, 10 cc. of normal monkey serum from monkeys 1 and 2 taken before those animals had been infected. Both cases came down, as the temperature charts show, with typical measles (fig. 3).

Persons F and G received 2 cc. of the mixture of infectious blood and physiologic salt solution, and 24 hours later received an injection with 20 cc. of a 1:1 dilution of monkey convalescent serum obtained from monkeys 4 and 11 two to three weeks after the fever had disappeared in those animals. Both persons remained free from symptoms (fig. 3).

The history of all these subjects regarding measles and any other disease was known without any doubt as they had lived since the first days of their lives under daily medical care.

CONCLUSIONS

The virus causing measles can be kept alive for several weeks outside the human system if blood of measles cases is drawn just at the beginning of the rash and diluted in the proportion of 1:7 to 1:10 with buffered salt solution containing the same number of anions and cations and showing the same P_H as blood. This mixture must be kept at a temperature 0 C.

Measles produced by inoculation in the skin begins earlier than natural measles or measles following an artificial infection of the mucous membranes of the respiratory tract, furthermore the symptoms are milder and of shorter duration. There seems to be an analogy between morbillisation against measles and variolation against smallpox.

Measles can be produced in human beings with material sterile from a bacteriological point of view, with sterile blood of a measles patient drawn at the right time, or with dilutions of such blood, which have passed through a Berkefeld filter. Sterile filtrates of nasal secretions collected in the preeruptive stage of the disease and diluted with physiologic salt solutions are also capable of producing measles.

Measles virus can be grown *in vitro* in culture mediums containing plasma from susceptible or immune persons which is diluted in a proportion of 1:6 or 1:7 with buffered physiologic salt solution. In order to keep the virus alive and to secure growth, it is necessary to associate it with living cells, such as slowly growing bacteria regularly found in cases of measles.

With sterile filtrates of cultures, reactions which are similar to measles can be provoked in human beings, the specificity of which can be proved by the fact that such persons are later immune against large amounts of infectious blood.

Monkeys (*Macacus rhesus*) can be made sick by injections of such material and the specificity of the reactions can be proved by the fact that their serum collected after the reaction can protect infected human beings against measles, while the serum of normal monkeys does not.

A GRAM-POSITIVE DIPLOCOCCUS IN HUMAN PROTOZOAL INFECTIONS

W. L. VOGEL

From the Clinical Laboratory, Riverside, and the General Hospital, Los Angeles, California

THE INCIDENCE AND DETERMINATIVE CHARACTERISTICS

Since the detailed account by Loesch¹ in 1875 attracted attention to the possible relationship of amoebae to dysentery, a vast amount of work has been done on the subject of intestinal protozoa in man. It is apparent from the clinical literature, that due to the wide variation in the picture presented by these protozoal infections many cases have gone unrecognized, being treated symptomatically. Probable reasons for the existing circumstance in this regard are first, that pathology teaches us to expect the extensive necrosis of the intestinal submucosa characteristic of the classical or acute tropical case, whereas, the clinician more frequently encounters the chronic type of infection, and limited to sigmoidoscopic examination sees an ulcerative condition rather uncertainly described as "amoebic colitis," "idiopathic ulcerative colitis," etc.; and second, that the exact nature of the injurious substances that may be produced by protozoa are little understood because of the difficulty encountered in attempting to grow these organisms in laboratory mediums. Consequently, the problem confronting the protozoologist is not unlike that encountered in attempting to explain the etiologic differences between the "common cold" and influenza, and coexisting factors are to be considered. Hypotheses have been advanced attempting to explain the peculiar toxemia and pathology of amoebiasis, but no report has been found concerning the bacterial flora common to the intestinal tract in these cases. It is with this phase of the matter that the following studies deal.

Since only those specimens submitted to the clinical laboratory primarily for parasitological examination are included in this study a relatively high percentage of protozoal infections is reported. For the same reason it is believed that a fairly representative bacterial flora likely to be found in stools and mucous excreta submitted for parasitological examination was encountered. The stools, mucous excreta, and in about 25% of the cases material obtained by the use of the sigmoidoscope were examined from 119 patients. An average of five specimens per patient were submitted, making approximately 570 examinations. All protozoa

Received for publication, June 11, 1927.

¹ Arch. Path. Anat., 1875, 65, p. 196.

were identified by the criteria of Kofoid,² and of Dobell and O'Connor³ with the Donaldson⁴ and iron-hematoxylin³ staining methods; material being stained by both methods routinely. All primary cultures for the study of the bacterial flora were made in 100 cc. quantities of a veal infusion broth adjusted to approximately P_H 7.6 to which 2 cc. of human blood was added. Transplants were made after 24 hours to methylene blue eosin agar (Bacto) and to blood agar plates. Our earlier studies were carried out with a 3% citrated human blood agar prepared from a nutrient agar base (Bacto) and poured into very thin plates; the recent work with 7.5% defibrinated rabbit blood agar prepared from a veal infusion base and poured to a depth of from 5 to 7 mm. in plates.

Identifications were made after 24 hours from the appearance of growth and discrete colonies, with stained preparations, and in a few instances with agglutination reactions. Transplants for the obtaining of pure cultures were made in veal infusion broth and agar respectively adjusted to P_H values of 7.6 and 7.8. Dilution methods and repeated platings were frequently necessary.

INTESTINAL FLORA OCCURRING IN 119 CASES

	Number of Cases		Number of Cases
Colon Group (<i>B. coli</i> , <i>B. aerogenes</i> , and others, culturally and morpho- logically similar to <i>B. coli</i> . Included in this heading are the indeter- minate, saprophytic gram-negative strepto-, and diplo-bacilli).....	111	Aerobic sporulating rods (<i>B. subtilis</i> and others)	20
<i>B. dysenteriae</i> (Flexner Type).....	2	Yeasts, molds, hypomycetes (species not identified)	9
<i>B. typhosus</i> (carrier).....	1	Sarcinae	4
<i>B. paratyphosus B.</i>	1	<i>Streptococcus</i> (alpha)*	11
<i>B. alkaligenes</i>	1	<i>Micrococcus catarrhalis</i> *	12
Lactobacillus group:		<i>Micrococcus tetragenes</i> *	4
<i>Acidophilus</i> forms and <i>B. bulgaricus</i>	73	<i>B. tuberculosis</i> * (not demonstrated culturally)	1
<i>L. hoas-oppleri</i>	4	Gram-positive rods, large (in stained preparations only: regarded as sporu- lating anaerobes)	21
<i>Streptococcus</i> (gamma), nonhemolytic.	22	Spirillae	14
Large gram-positive diptheroid rods (nonsporulating)	18	Gram-positive diplococcus	54

* Probably swallowed respiratory organisms.

NOTE:—The only day to day variation of bacterial flora observed was among the lactobacilli and those organisms regarded as swallowed respiratory bacteria.

Material from sigmoidoscopic investigations was cultured in a semisolid cooked 3% human blood medium containing 1½% agar. Stab inoculations were made with the original cotton tipped wooden applicators bearing the inoculum.

With striking regularity a gram-positive organism, morphologically a diplococcus, occurred in addition to the normal bacterial flora in 54 of the 59 cases in which the pathogenic protozoal species were demonstrated as presented in the tabulation. This bacterial entity was not observed in association with *E. coli*, *E. nana* nor the *Trichomonas*, except when these protozoa occurred in association with *E. dysenteriae*, *Councilmaniana laffleuri*, *Chilomastix mesnili* or *Lamblia intestinalis*. The diplococcus was not observed in the absence of protozoan forms. No other bacterial species occurred consistently in parallel with these pathogenic protozoa

² Kofoid, C. A.; Kornhauser, S. I., and Swezy, Olive: Arch. Int. Med., 1919, 24, p. 35; Kofoid, C. A., and Swezy, Olive: Univ. of Calif. Pub. Zool., 1920, 20, p. 117.

³ The Intestinal Protozoa of Man, 1921.

⁴ Lancet, 1917, 2, p. 571.

except the gram-negative rods of the colon group. The lactobacilli were found in about one-half of these cases.

In animal inoculations, strains of this diplococcus in pure culture produced definite lesions in the intestinal tract and nowhere else, from which the organism could frequently be recovered in pure culture. This elective localization of the organism with the production of lesions attracted attention to the possibility of a symbiotic rather than casual relationship existing between it and those protozoal forms which we have come to regard as pathogenic. Of further interest is the non-occurrence of this relationship between the pathogenic protozoa and the bacterial entity in 5 cases.

Of the 59 cases in which those protozoa commonly regarded as pathogenic were found, *E. dysenteriae* was identified in 49 cases; *Chilomastix mesnili* in 20 cases; *Councilmania lafleuri* in 1 case;

THE DIPLOCOCCUS-PROTOZOA RELATIONSHIP AND THE ASSOCIATION OF PROTOZOAL SPECIES
IN 59 CASES

Protozoa	Number of Cases	
	Examined	Diplococcus Present
<i>E. dysenteriae</i> (unassociated).....	18	16
<i>E. dysenteriae</i> (<i>E. coli</i> , <i>E. nana</i> and <i>Trichomonas</i> associated)...	17	17
<i>E. dysenteriae</i> (<i>Chilomastix mesnili</i> associated).....	14	12
<i>Chilomastix mesnili</i> (unassociated).....	4	4
<i>Chilomastix mesnili</i> (<i>E. coli</i> , <i>E. nana</i> and <i>Trichomonas</i> associated)	2	2
<i>Councilmania lafleuri</i> (unassociated).....	1	1
<i>Lambliia intestinalis</i> (<i>Giardia</i>) (unassociated).....	2	2
<i>Lambliia intestinalis</i> (<i>Giardia</i>) (<i>E. coli</i> associated).....	1	0

In each case protozoological findings are a summary of information derived from all of the specimens from that patient.

Lambliia intestinalis (*Giardia*) in 3 cases. The association of these forms is shown in detail, together with the diplococcus-protozoa relationship (tabulation). Of the 119 cases studied some protozoal form was found in 79, or 67.2% of these cases; the so-called pathogenic protozoa in 59, or 49.5%; the nonpathogenic protozoa in 20, or 16.8%; the diplococcus in 54, or 45.3%. In the protozoal infections (pathogenic) the diplococcus occurred in 54 (91.5%) of the cases and was absent in 5 (8.5%).

Of the 49 cases in which *E. dysenteriae* was identified, but 1 case was characterized by an acute dysentery.

It will be observed that *E. coli*, *E. nana* and *Trichomonas* were found in association in some of the above cases. The 20 cases referred to, however, are those in which one or more of these species occurred but not in association with *E. dysenteriae*, *Councilmania lafleuri*, *Chilomastix mesnili*, nor *Lambliia intestinalis* (*Giardia*).

No opportunity has been had to study the geographical occurrence of this parallelism or symbiosis observed in Southern California. However, Bargen and Logan,⁵ whose work appeared just at the completion of this series, report as the etiologic factor in idiopathic ulcerative colitis an organism seemingly identical with the diplococcus described. These workers state that because of the fact that in many of their cases there was no reason to suspect an amoebiasis, this phase was not particularly stressed in their investigations. They report the finding of amoebas in 12% of their cases.

In six cases in which the *E. dysenteriae* was readily demonstrated in the feces and mucous excreta it was possible to isolate the diplococcus only after the repeated culturing of material obtained with the sigmoidoscope. In the 5 protozoa-positive cases in which the diplococcus was absent, repeated sigmoidoscopic material was submitted for examination, which together with the stools were consistently negative for the diplococcus. Ordinarily freshly isolated diplococcus cultures contained a gram-negative rod and in many instances difficulty was experienced in obtaining pure cultures for study. Lesions produced by such mixed cultures were definitely duplicated in each instance with the same strain of the diplococcus in pure culture after such animal passage. Animal passage was found to be a valuable means of obtaining the organism free from contamination and in a number of instances pure cultures were readily isolated by the subcutaneous inoculation of small laboratory animals with minute quantities of suspicious material emulsified in 1 cc. of normal salt solution. This method was found to be of particular value where but an occasional gram-positive coccus was observed in stained preparations which did not appear in primary cultures at all due to the rapid and luxuriant growth of the gram-negative bacilli present.

A pure culture study of the morphologic and cultural as well as the biochemical characteristics of the diplococcus was undertaken, the findings presented being based upon the parallel study of 27 strains of the organism under identical conditions, using similar mediums and methods.⁶ The specific name, *Diplococcus denitrificans*, is suggested by the writer for this bacterial species. The determinative characteristics of the diplococcus are as follows:

Morphology: Morphologically the organism is a diplococcus somewhat resembling *Diplococcus pneumoniae* and occurs in spherical, oval or lancet-shaped forms in pairs and tetrads. It is characterized by a marked variation in size, the individual coccus ranging from 0.4 to 1.0

⁵ Arch. Int. Med., 1925, 36, p. 818.

⁶ Manual of Methods for Pure Culture Study of Bacteria, Society of American Bacteriologists, 1923.

micron in diameter. It is gram-positive, with gram-negative forms only in very old cultures. The organism is not encapsulated, but an irregular gelatinous, transparent pseudocapsule or binding material is observed in some strains. Flagella have not been demonstrated. It is bile insoluble.

Oxygen Requirements: The organism is a facultative anaerobe, a rapid luxuriant growth being obtained under aerobic conditions and a scanty to moderate growth in anaerobic cultures.

Classification According to Optimum Conditions: A more luxuriant growth is obtained at 37 C. than at 25 C., and a more luxuriant growth on solid than on liquid mediums. The latter preference is the more marked. According to the classification of the Society of American Bacteriologists the organism, due to its preference for incubator temperature and solid mediums falls in series 3.

Relation to Reaction of Medium: Optimum H-ion Concentration about PH 7.8. Limits of PH for growth, from 5.2 to 8.2.

Chromogenesis: No pigment production is observed in broth, glycerol broth, agar, gelatin or potato.

Food Requirements: Excellent growth is obtained on ordinary mediums.

Ferment Action: Acid formed in dextrose, lactose sucrose, maltose, levulose and galactose. No acid formed in dextrin, salicin, inulin, xylose, dulcitol and mannitol.

No gas is produced in any of the above carbohydrates in which acid production takes place. Acid is produced in milk and the medium curdled. No variation in the ability of any of the strains investigated, to ferment the above carbohydrates was observed over a period of three months following isolation.

Proteolytic Action: Gelatin is liquified in seven days by most strains, an occasional strain, however, requiring a slightly longer period of time for the production of the liquifying enzyme, in sufficient concentration. Loeffler's blood serum is not liquified. Milk is not digested.

Reduction of Nitrates: Nitrates are rapidly reduced to nitrites with the evolution of large quantities of gas (free nitrogen) within 48 hours.

Diastatic Action: Starch is not hydrolyzed, nor is diastase produced.

Aromatic Products: Indol is not produced. The cholera-red reaction is negative. Acetyl-methylcarbinol (Voges-Proskauer reaction) is not produced.

Hydrogen Sulphide Production: Hydrogen sulphide is produced, lead acetate medium and test paper being blackened, slowly.

No surface growth and but a transient clouding of medium, clearing after a few days is observed in broth cultures. The sediment is compact and scanty. Cultures are odorless.

On nutrient agar growth is abundant, filiform, contoured, raised and of butyrous consistency. It is white, opaque, dull, nonphotogenic, and nonfluorescent. The medium is unchanged. Circular, smooth, umbonate (button-like) colonies from 2 to 4 mm. in diameter, develop rapidly within 24 hours. Colonies are of even margin; the internal structure finely granular.

On blood agar the beta type hemolysis (Brown) is observed in 24 hours. Deep and surface colonies are gray in color. On Loeffler's blood serum a pure white growth scarcely discernible from the surrounding medium occurs. Growth on potato is scanty, filiform, flat, dull, whitish, granular, adherent and dry.

A series of laboratory animals were immunized with strains of the diplococcus, precipitins, complement-fixing antibodies, and agglutinins being identified in the resulting immune serums. Precipitins and complement-fixing antibodies were demonstrated prior to the appearance of agglutinins in the order named. Three immunologic types of the diplococcus have been positively identified, the absorption and precipitin tests confirming the agglutination reactions. With types 1, 2, 3 and 4 pneumococcus antigens in the presence of a polyvalent diplococcus immune serum no agglutinations occurred. The antigens of seventeen strains of hemolytic streptococci isolated from acute processes and representing at least two definite immunological types, corresponding to the mannitol-nonfermenting strains of Dochez ⁷ showed no agglutinations in the presence of the diplococcus immune serum.

⁷ Dochez, A. R.; Avery, O. T., and Lancefield, R. C.: J. Exper. Med., 1919, 30, p. 179.

From the fact that the readily demonstrable hemolysin of the diplococcus would not alone account for the ulcerative lesions produced in these infections, it is believed that the injurious substance is a metabolic product. As the coccaceae are characterized by a complex metabolism, involving the utilization of amino acids or carbohydrates, the energetic reduction of nitrates by the diplococcus with the liberation of large volumes of nitrogen in vitro is held as significant in view of the fact that the organism is not strongly saccharolytic.

PATHOGENICITY

Series 1.—A series of large, healthy guinea-pigs were inoculated intraperitoneally from the 27 strains of the diplococcus just described. Each strain of the organism was subjected to anaerobiasis to satisfy ourselves that we were working with pure cultures throughout the subsequent animal work. Each guinea-pig received the 24-hour growth from a veal infusion agar slant emulsified in 1.5 cc. physiologic salt solution. Necropsy was performed on each animal in 21 days. A marked elective localization⁸ of the injurious effects of the organism for the intestinal mucosa was observed. Lesions varying in severity from minute pinpoint ulcers of the mucosa to extensive submucous hemorrhages in one case, were produced in 14 animals (51.8%). The diplococcus was recovered in practically pure culture from the lesions, and from the feces found in the colon at necropsy in 10 or 76.9% of those cases in which no lesions were produced. From the fact that pathology was produced in but a little over half of the 27 animals inoculated it was concluded that either a natural and varying immunity occurred in guinea-pigs, or that these innocuous strains were of low-grade virulence, due to the chronic condition from which they were isolated.

Series 2.—Five healthy guinea-pigs averaging 400 grams in weight were inoculated in parallel with a strain of the diplococcus recovered from the accumulated feces in the colon of one of the above animals in which no lesions were found. The technic of inoculation and the amount of inoculum was identical with that used in the preliminary inoculations and is used throughout these experiments. Necropsy was again performed in 21 days in each instance and the observations tabulated as follows.

GUINEA-PIGS	CHARACTER OF STOOLS	LESIONS AT NECROPSY
1 and 2.....	Normal	None
3 and 4.....	Fluid	No lesion in 3; mesenteric lymph nodes enlarged and acutely hemorrhagic, in 4
5	Fluid, streaked with blood and mucous	Duodenal ulcerations 2 mm. in diameter; pinpoint lesions in cecum to rectum.

The diplococcus was isolated in pure culture from the lesions in animals 4 and 5 and from the feces in animals 2 and 3, but not from the feces of animal 1, or lesions in 40% of the 5 animals.

Series 3.—To determine experimentally to what degree the varying resistance of individual animals could be overcome by repeated animal passage and to what degree the injurious effects of the organism could be increased by animal passage, another series of guinea-pigs of the same average weight (400 gm.) were inoculated from transplants of cultures from the lesions of animal 5, by the same

⁸ Rosenow, E. C.: J. Dent. Res., 1919, 1, p. 205.

method. Surviving animals were subjected to necropsy at ten days following inoculation, and the diplococcus isolated in practically pure culture from all animals (tabulation) in which lesions were found.

PATHOLOGY PRODUCED BY STRAINS OF DIPLOCOCCUS FOLLOWING ANIMAL PASSAGE

Guinea-Pigs	Character of Stools.	Lesions at Necropsy
1	Fluid	Minute, scattered ulcers in colon
2	Diarrhea	Minute punched-out ulcers in cecum
3	Bloody diarrhea	Duodenal ulcerations, hyperplasia of mesenteric lymph nodes. Extensive submucous hemorrhages. Died on 5th day
4	Fluid; mucous.....	Minute ulcers, lower half of cecum (yellow pustulate). Scattered granular lesions ileum and upper cecum
5, 6 and 7.....	Normal	Hyperplasia mesenteric lymph nodes (5); minute, scattered ulcers in colon (6), and 1 hemorrhagic ulcer 4 mm. in diameter in duodenum. Nearly perforating* (7)
8 and 9.....	Normal	None
10	Fluid for 3 days.....	None

* Probably due to perforation of duodenum at time of inoculation.

As in the earlier series the inoculum was in each instance a salt solution suspension of the living organisms emulsified from solid mediums, as it was desired to study only the injurious effects of the organism in vivo at this time, uncomplicated by the toxicity of a peptone medium and such poisonous substances as might be produced in vitro in fluid medium.

Throughout these experiments guinea-pigs were fed exclusively on carrots, and carrot tops and given a minimum amount of water.

Guinea-pigs 4 and 5 of series 1 and in the subsequent series all in which lesions were found were drowsy, refusing to move unless disturbed, the eyes presenting a glassy appearance indicative of an increased body temperature. Fever, chills, rigidity and the appearance of acute sickness were observed in some animals, corresponding to the degree of cellular destruction.

Guinea-pig 2 of series 3 was suffering from an iritis and was totally blind the day prior to necropsy, that is on the 9th day following inoculation. In the case of animal 10, the diplococcus was isolated from the feces 8 hours following inoculation.

Series 4.—Five guinea-pigs were inoculated, animals 1, 2 and 3 intraperitoneally, and animals 4 and 5 intramuscularly from the lesions in guinea-pig 5 of series 2 with the results tabulated as follows.

GUINEA-PIGS	CHARACTER OF STOOLS	LESIONS AT NECROPSY
1	Fluid; frequent.....	Extensive intestinal lesions. Died in 15 hours
2, 3, 4 and 5.....	Bloody	Extensive intestinal lesions. Died in 19, 24, 36 and 48 hours, respectively

All animals showed marked clinical symptoms as described, setting in almost immediately after inoculation. The diplococcus was demonstrated culturally on mediums inoculated from the feces 8 hours following inoculations. It is believed that the building up of virulence observed from these experiments is significant and worthy of consideration in the study of the infectiousness of amoebiasis, particularly when the bacteria ingestion of protozoa is considered.

Series 5.—A further experiment based on the necropsy findings in ten guinea-pigs, five of which were inoculated intramuscularly to determine whether or not a local reaction would occur at the site of inoculation, from strains of the diplococcus isolated from five patients with chronic amoebiasis in which punched-out

and pustular lesions were found in the sigmoid. The remaining five animals were inoculated intramuscularly with five strains of the diplococcus isolated from the feces of patients in which no lesions were reported in the colon.

GUINEA-PIGS	CHARACTER OF STOOLS	LESIONS AT NECROPSY
1	Normal	Pinpoint lesions in colon; congestion mucosa of jejunum
2	Fluid	Mesenteric lymph nodes enlarged and acutely hemorrhagic
3	Fluid, streaked with blood and mucous	Pinpoint lesions in cecum to rectum
4	Normal	Minute, scattered pustular lesions in cecum
5	Fluid	Scattered granular lesions in ileum and cecum. Hyperplasia mesenteric lymph nodes
6, 7 and 8.....	Normal	None
9	Fluid	None
10	Fluid	Punched-out pinpoint lesions in lower ileum and cecum

Necropsy was performed on each animal after 21 days. In no instance, throughout the series was any local reaction observed at the site of inoculation, beyond a slight edema in several cases, including those guinea-pigs receiving intramuscular inoculation. The diplococcus was isolated from the feces of animals 5 to 9 of series 5 as well as from the lesions in animal 10. The strains with which animals 1 and 2 were inoculated had been grown on laboratory mediums for nine generations (transplants) prior to inoculation. None of the strains studied failed to produce more or less fulminating lesions after repeated animal passage even after having been grown on laboratory mediums for long periods of time. Limited experiments with inoculation by feeding conducted paralleling in the pathology produced, that of intraperitoneal and intramuscular inoculations.

Series 6.—A short series of rabbit inoculations were undertaken for additional information as to the nature of the pathology produced in experimental animals and to if possible coordinate the writer's findings with those of Bargen and Logan. Rabbits were fed exclusively on the vitamin-free diet of rolled barley and water of McCarrison.⁹ Consequently, in those animals reported as being symptom-free, it will be understood that the statement has reference to symptoms other than the malaise characteristics of the dietary deficiency disease intentionally produced. Symptoms in rabbits were almost identical with those produced in guinea-pigs.

Intravenous inoculation was resorted to, the first five rabbits reported in the tabulation receiving the same quantity of inoculum as the guinea-pigs; the remaining five receiving approximately twice the amount. With the exception of animal 6 of this series, which died ten days after the inoculation, necropsies were routinely performed at 21 days. Elective localization of infection in rabbits inoculated with strains isolated from sigmoid lesions was shown.

RABBITS	SYMPTOMS	LESIONS AT NECROPSY
1	Absent	Few punched-out, pinpoint ulcers in cecum
2	Feces blood streaked.	Minute abscesses of mucosa upper jejunum and lower ileum
3	Absent	Congested patches in cecum 1 cm. in diameter
4	Absent	Furred over veins in cecum
5	Marked (hemorrhage)	Empyema of gallbladder, submucous hemorrhage, lower ileum and cecum
6	Feces fluid after 7 days	Hyperplasia mesenteric lymph nodes (acutely hemorrhagic)
7	Absent	Few punched out, pinpoint ulcers in ileum, 3 lesions 1 mm. diameter in cecum, yellow pustular heads
8, 9 and 10.....	Absent	Absent

⁹ Studies in Deficiency Disease, 1921, p. 270.

In the case of animal 2, in addition to the intestinal involvement a process resembling an early osteoarthritis was demonstrated in the middle joint of the left hind leg. The diplococcus was isolated in pure culture from the abscesses in the jejunum and ileum and in stained preparations of material from the joint an occasional gram positive organism resembling the diplococcus was found. Cultures from the joint, however, both aerobic and anaerobic, were found upon subsequent examination to be sterile. No swelling nor enlargement of the joint had been observed prior to inoculation. It is of particular interest that an indeterminate protozoal form, somewhat resembling *E. dysenteriae* was demonstrated in the stools and intestinal contents of this animal; a *Chilomastix*-like flagellate having been demonstrated previously in the stools of guinea-pig 2 of series 3.

In 1922 Byea and Nathan¹⁰ reported the finding of *Micrococcus tetragenus* in a case of osteoarthritis, and inasmuch as morphologically the diplococcus, in its tetrad presentation easily mistaken for the *Micrococcus tetragenus*, it is thought that these workers may have been the first to observe the diplococcus in this type of case in man, the removal of such an organism supposedly being accomplished by the migration of a protozoal laden with bacteria from the intestinal tract.

The marked elective localization of the lesions of the diplococcus, to the intestinal tract is held as being particularly significant when linked with the coexistence of the diplococcus with the pathogenic protozoa. Blood stream invasion was noted in all of the animals of series 4.

METABOLISM

Three typical strains of the diplococcus were isolated in pure culture by the usual laboratory methods from sigmoid lesions. In two of these cases the *Entamoeba dysenteriae* was present in the feces and in the third case, *Entamoeba dysenteriae* and a species of trichomonad. Protozoa were identified in stained preparations by the usual iron-hematoxylin method. Each strain was inoculated in a 100 cc. quantity of nutrient broth, P_H 7.0, as seed cultures for the following experiments.

A series of flasks each containing exactly 1000 cc. of sterile broth containing 10 gm. peptone, 5 gm. sodium chloride, and 10 gm. dextrose per liter, were adjusted with a LaMotte H-ion comparator set through the H-ion concentration range from P_H 4.8 to 8.4 at intervals of two tenths. Standard buffer solutions of these values and the indicators, bromcresol purple, bromthymol blue and phenol red were utilized, adjustments being made with normal solutions of hydrochloric acid and sodium hydroxide. A control flask was included, duplicating the concentration of that in the series adjusted to P_H 7.0. In no instance was the volume increase due to the addition of acid or base in excess of 5 cc. per liter of medium, or 0.5% of the total volume. It was found impracticable to adjust mediums in quantities of less than 1 liter.

From the supply flasks so prepared, a 100 cc. quantity of broth was poured into three 200 cc. flasks at each of the nineteen P_H values throughout the range from P_H 4.8 to 8.4 and from the P_H 7.0 control medium. All flasks poured at

¹⁰ Ann. Med., 1922, 2, p. 193.

this time were tightly plugged with cotton, covered with gauze and thin, sheet rubber and autoclaved at 15 pounds for 10 minutes. Following sterilization these quantities of mediums were placed in the incubator for 24 hours to insure sterility. From the seed cultures previously mentioned a flask of dextrose broth was inoculated from each of the three strains at each value throughout the range P_H 4.8 to 8.4 including the control mediums. Inoculations were accomplished with sterile graduated pipets, each flask receiving 0.1 cc. of the respective seed cultures. Cultures were incubated for 7 days at 37.5 C. and examined. The results were identical with each strain, and growth was observed throughout the range P_H 5.2 to 8.2, no growth being observed at P_H 4.8, 5.0 or 8.4. From P_H 6.2 to 8.2 the endpoint of reaction of cultures was P_H 6.2. From P_H 5.2 to 6.0 the reaction of cultures was found to be identical with the P_H values of the medium throughout this range prior to inoculation. The endpoint reaction of the control cultures was found to be P_H 6.2.

Cultures were thoroughly shaken for 5 minutes to insure an even bacterial suspension and 10 cc. quantities accurately pipetted into a series of Babcock vaccine centrifuge tubes from the cultural set-up outlined above for each of the three strains of the organisms under investigation. Tubes were centrifugalized for 30 minutes at 2400 r. p. m. and readings taken from the capillary columns. Based upon the actual amount of cellular sediment by volume, obtained after uniform handling by this method, the limits of growth were found to be from P_H 5.2 to 8.2; the optimum range from P_H 7.6 to 8.2. A parallelism in optimum and endpoint conditions in the growth of the diplococcus and of intestinal protozoa in laboratory medium is called attention to. Kofoed and Wagener,¹¹ Boeck,¹² and Craig¹³ point out that the optimum concentration for the growth of *Entamoeba dysenteriae* falls between P_H 7.8 and 8.0. According to Kofoed and Wagener the final P_H of their amoeba cultures is from P_H 6.2 to 6.4.

As previously reported the diplococcus is characterized by an energetic nitrate reduction in vitro and a series of experiments were undertaken to substantiate this finding. Ten days from the time of inoculation of the dextrose broth cultures, nitrogen determinations were made on their filtrates. Similar determinations were made on the residual uninoculated medium at each H-ion concentration throughout the range being studied, using the method of Folin¹⁴ as recommended for non-protein nitrogen determinations on blood filtrates. Findings reported, however, are in terms of total nitrogen, since the protein elements were not precipitated, and the tests were conducted on unaltered fluid mediums before and after inoculation. Determinations were made on 5 cc. quantities of a 1:20 dilution of medium and filtrates—a dilution applicable to colorimetric methods. In the accompanying chart, nitrogen readings are recorded relatively, that is, on the basis of nitrogen per 100 cc. of 1:20 dilution. A variation in the nitrogen content of the

¹¹ Univ. Calif. Pub. Zool., 1925, 28.

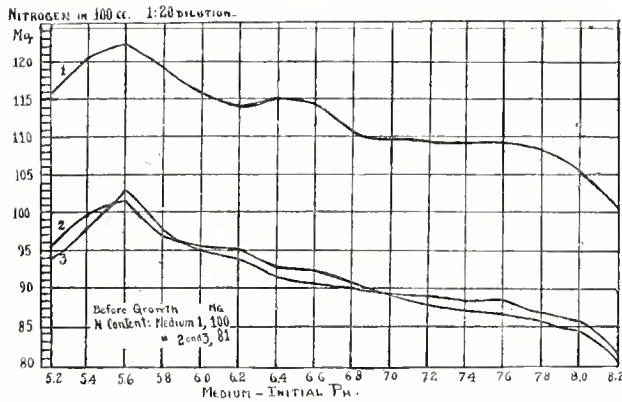
¹² Am. J. Hyg., 1923, 5, p. 371.

¹³ Am. J. Trop. Med., 1926, 6, p. 5.

¹⁴ Laboratory Manual of Biological Chemistry, 1923.

residual sterile broths was noted, due to the small additions of acid or base in adjustments of their reactions. Readings were from 77 mg. to 86 mg. per 100 cc. of 1:20 dilution of medium, or an average of 80.94 mg. Due to the nature of the findings in the inoculated broths this variation was neglected and 81 mg. taken as the base line for curves 2 and 3. Neither the carbohydrate, nor the chloride content of the medium seemed to interfere with the determinations.

Identical experiments were repeated in the absence of carbohydrate with a peptone medium, omitting the dextrose, and with this plain peptone medium to which .01% potassium nitrate was added. In the absence of fermentable carbohydrate, at the expiration of ten days following inoculation, the reactions of these mediums were practically



Total nitrogen determinations for cultures of the diplococcus (average of 3 strains) in nitrate-peptone (1), dextrose-peptone (2), and plain peptone mediums with indicated PH values.

unchanged. The average nitrogen content of the sterile nitrate-peptone medium was found to be approximately 100 mg.

The rise in N content at the beginning of the acid range (chart) is followed by a decline with a marked drop through the band of H-ion concentrations optimum to the growth of the diplococcus and to intestinal protozoa.

Amino acid determinations on sterile and inoculated plain peptone medium throughout the range suitable for the growth of the diplococcus, after the method of Folin were indicative of a synthesis of the liberated nitrogen into a complex metabolic product. Determinations were made at selected concentrations of media throughout the acid and alkaline range. The amino acid content showed a marked rise within the optimum range for the growth of the diplococcus. Inasmuch

as determinations were not made at each concentration of medium, graphic presentation of the findings are not recorded.

Kofoid and Wagener¹⁵ have studied the effect of a group of drugs commonly used in the treatment of human protozoal infections with reference to their germicidal potency for amoebae grown in vitro. Their findings substantiate the successful clinical administration of a number of these drugs, and particularly of emetine hydrochloride and stovarsol. The effects of these two drugs on the diplococcus was studied in vitro at dilutions approximating those at which the workers mentioned found them to be toxic for *Entamoeba dysenteriae*. Luxuriant growth of the diplococcus occurred in medium seeded with cultures which had been exposed to fifty times their volume of 1:1,000, 1:2,000, 1:5,000 and 1:10,000 dilutions of emetine hydrochloride and stovarsol for periods of time ranging from 2.5 minutes to 24 hours. Kofoid and Wagener have pointed out in their report that no visible reduction in the bacterial content of their cultures containing these drugs, as compared to the controls could be detected. Their investigations were conducted with *Entamoeba dysenteriae*, isolated from feces in mixed cultures with bacterial flora.

SUMMARY

A hemolytic gram-positive diplococcus differing immunologically from the hemolytic streptococci and from the diplococcus of pneumonia, was found in the intestinal flora of 54 of the 119 cases of suspected amoebiasis. Protozoa were present in 79 cases, with pathogenic species in 59, and in these the diplococcus was present in 54 (91.5%). Intestinal lesions were produced in inoculated animals and the diplococcus recovered in turn from these lesions.

The optimum H-ion concentration for growth of the diplococcus approximates that reported by other authors for pathogenic protozoa in vitro, and maximum denitrification takes place at this P_H range. Emetin hydrochloride and stovarsol are not germicidal for the diplococcus and the suggestion is made that this organism may be a contributory factor in the pathology and peculiar toxemia accompanying the protozoal infections, especially in those cases which fail to respond to treatment.

¹⁵ Univ. Calif. Pub. Zool., 1925 28, II.

BACILLUS SORDELLII, A CAUSE OF MALIGNANT EDEMA IN MAN

IVAN C. HALL AND JOSEPH P. SCOTT

*From the Department of Bacteriology and Public Health, University of Colorado Medical School,
Denver, and the Veterinary Department, Kansas State Agricultural
Experiment Station, Manhattan*

The fact that none of the highly putrefactive bacteria is able to cause disease by the penetration of animal tissues suggests that if a putrefactive organism displays pathogenicity it must do so by virtue of a soluble toxin. This is certainly the case with *B. botulinus* and *B. tetani* and probably with *B. histolyticus*. It appears also to be true of the highly pathogenic anaerobe recently discovered in 2 out of 11 cases of acute edematous wound infections in South America by Sordelli¹ and described by him under the name "*Bacillus oedematis sporogenes*."²

This germ combines the pathogenic properties of *B. oedematiens* (*B. novyi*) with some of the morphologic and cultural properties of *B. sporogenes*; it produces the massive edema without hemorrhage, gas, or histolysis, of the former in animals, yet shows the proteolytic tendencies of the latter in culture mediums. In this it resembles the "Fahr" culture which Zeissler described as a pathogenic strain of *B. sporogenes*. But Sordelli and Gez³ showed that the Fahr culture was really a mixture of *B. sporogenes* (Metchnikoff) and *B. oedematiens* (*B. novyi*), and the same suspicion naturally attached to his own cultures but he was never able to prove any impurity and concluded, as we believe, correctly, that he was dealing with a new species.

Dr. Sordelli kindly forwarded his two strains to the senior author in August 1924 with a request that his findings be checked if possible. We were able to do so in full, excepting for motility, for one strain only; the other never showed any pathogenicity, although morphologically and culturally, the two were similar. It is therefore the purpose of this contribution to confirm the identity of this new species and point out its relation to the better known anaerobes. We desire also to

Received for publication, July 9, 1927.

¹ Rev. Asoc. med. Argentina, 1922, 35, p. 217; Compt. rend. Soc. de Biol., 1922, 87, p. 838.

² (a) Rev. Asoc. med. Argentina, 1923, 36, p. 219; (b) Compt. rend. Soc. de Biol., 1923, 89, p. 53; (c) Rev. del Inst. Bact., Dpt. Nac. d. Hig., 1923, 3, p. 1.

³ Compt. rend. Soc. de Biol., 1924, 91, p. 1033.

suggest the substitution of a binomial, "*Bacillus sordellii*," to replace the trinomial, "*Bacillus oedematis sporogenes*," which is not only invalid according to the rules for naming species,⁴ but also confuses this germ with two other distinct species. We do not believe in the wholesale erection of monumental specific names but in the present instance it seems to be the best solution of a taxonomic tangle.

Morphology.—*B. sordellii* is a gram-positive rod, 1.2 to 1.5 μ and 1.5-8.0 μ long with rounded ends. Filaments as long as 45 μ occur occasionally. It is described as motile but we have never been able in numerous examinations to detect any motility except in very young cultures and then only in a very few organisms. In 24-hour cultures in brain medium the rods are either single or paired, or may form short chains of 3 to 4 cells. A few subterminal spores, which swell the rods, may be seen. In cultures 3 to 5 days of age, more chains are seen, and a large number of rods containing oval subterminal spores. Numerous free spores are also present, many of which have considerable adherent vegetative protoplasm. Spores are best seen in smears prepared from blood agar slants in tubes from which the oxygen has been absorbed by alkaline-pyrogallol.

Cultural Characters.—*B. sordellii* grows readily in all the mediums and devices regularly used in anaerobic work.⁵ In brain medium gas and turbidity appear overnight. The brain is not blackened except after many days, but if a piece of iron wire be added before inoculating, it blackens within 24 to 48 hours. Both in brain and other mediums an odor suggestive of boiled onions develops. Old cultures in brain medium show white crystals resembling those of tyrosine but the crystals have not been identified chemically.

There is no trace of growth on solid mediums, such as agar or rabbit blood agar slants, except when special means are taken to absorb oxygen; the organism is a strict obligate anaerobe. Under alkaline-pyrogallol, small dewdrop-like, mildly hemolytic colonies are produced on blood agar. In a few days these become somewhat flattened with raised opaque centers and spreading margins.

Since there was at least a suspicion of impurity, we naturally paid special attention to the form of colonies in deep 1% meat infusion agar. These were of five types: a solid, lenticular, spherical or irregularly shaped colony having a light fluffy border; a granular colony composed of a number of small solid colonies; a fragmented colony composed of a solid center with thick projections; a multiple disc colony having 4 to 8 semicircular discs attached at their centers, apparently a modification of the fragmented type; and a fluffy or feathery colony having a solid center and feathery projections in all directions. Such cultures gave a strong impression that we were dealing with a mixture of several species but we wish to emphasize here that while identity of colony form in deep anaerobic cultures is strong presumptive evidence of purity, one should never be led to place implicit reliance on this criterion because there are distinct species whose colonies are so much alike as to be practically indistinguishable, or one might have two species associated of which the requirements of only one were met in the medium, so that an appearance of purity would be due to the failure of one to grow. It is equally unsafe to postulate impurity on a dissimilarity of colonies in a tube of deep agar because a pure culture may give rise to colonies of widely differing types, even in the same tube. The reasons for this are not

⁴ Buchanan: General Systematic Bacteriology, 1925.

⁵ J. Infect. Dis., 1920, 27, p. 576.

well understood but there is no doubting the fact. In some cases age of individual colonies accounts for the differences; solid colonies not infrequently fragment or become fluffy as they grow older. Arrested development of young colonies due to accumulation of growth products produced by older colonies probably accounts for some of the observed differences. If we assume that not all colonies start growing at the same time, we may account for some of the differences by supposing that the younger colonies are actually growing in a different hydrogen-ion concentration than that in which the older ones grew. In other cases we seemed to be dealing with true mutations, or dissociations—as they are called by Hadley;⁶ the senior writer has already called attention to such a case in a strain *B. tetani*⁷ and was interested to find the phenomenon similarly interpreted by Heller⁸ who studied the same culture independently. We can obviously recognize such mutations as such, only when they do not affect important features of our definitions of species.

Sordelli noted the pleomorphism of colonies in his cultures and was duly puzzled by it, as is everyone who encounters the phenomenon. We attempted to divide *B. sordellii* into two types by repeatedly picking either solid or feathery colonies from deep agar cultures, and thought for some time that we had succeeded, but prolonged study showed that the colonial characters are quite unstable.

A culture derived from a solid colony produced only solid, granular or fragmented colonies when reinoculated into deep agar. No feathery colonies were found in any of twelve successive agar generations made during the course of a month. After the seventh transfer the "solid" strain was passed through a guinea-pig. The recovered strain produced only "solid" colonies during four successive transfers on agar.

Comparable results were obtained with a culture derived from a feathery colony, which produced only feathery colonies during twelve agar transfers and also after guinea-pig passage.

The age of the cultures in brain medium from which the agar cultures were prepared had no effect on the form of the colonies produced, although young cultures seemed to produce better and larger colonies. But the types were the same from cultures in brain medium incubated for 15, 24, 48, 72, or 96 hours. The reaction of the agar also had no effect on the form of the colonies. A series of tests were made in 1% meat infusion agar with different hydrogen-ion concentrations: P_H —4.5, 5.0, 5.5, 7.0, 7.5, 8.0, 8.5; only "solid" colonies were produced from the "solid" strain and only "feathery" colonies from the "feathery" strain. The limits of growth were found to be P_H 5.5 to P_H 8.0 for the "solid" type, and P_H 5.5 to P_H 7.5 for the "feathery" type.

⁶ J. Infect. Dis., 1927, 40, p. 1.

⁷ Ibid., 1922, 30, p. 445.

⁸ Ibid., p. 33.

The morphologic, proteolytic, fermentative and pathogenic properties of both the "solid" and "feathery" daughter cultures were identical with each other and with those of the parent culture, excepting that the "feathery" culture seemed to be slightly more vigorous in growth than the "solid" culture. Yet, when the colonial characters of both the "solid" and "feathery" daughter cultures were again tested after ten months storage at 4 C, it was found that both types of colony appeared among the daughter colonies from each culture, suggesting that whatever properties of bacterial protoplasm may be responsible for colonial form, are, in this strain at least, most unstable. But the cultural and pathogenic characters showed no change in either case from the parent cultures and were identical.

The proteolytic properties of *B. sordellii* must be regarded as less marked than those of *B. sporogenes*. For example, brain medium made with Difco peptone, which contains some free iron⁹ is blackened within a few days by *B. sporogenes*, but only after many days by *B. sordellii*. But if a piece of iron wire be added the medium becomes black within 24 hours. Gelatin is liquefied rapidly and a black sediment forms in the bottom of the constricted tube below the marble seal. Loeffler's coagulated blood serum medium, which is almost completely digested by *B. sporogenes* in 48 hours, is definitely but more slowly and incompletely digested by *B. sordellii*. The reaction in milk differs slightly from that of *B. sporogenes* in that no real clot is formed but only a flocculent coagulation followed on the second or third day of incubation by gas production and slow liquefaction, which in our tests was not complete even after two weeks.

A curious reaction was observed in Petroff's gentian violet egg medium under alkaline pyrogallol. After incubation for 24 hours bright yellow colonies 0.5 mm. in diameter appeared, surrounded by a zone of lavender color which contrasted sharply with the purple color of the medium. On the following day it was evident that these lavender zones were merely the beginning of a very complete decolorization of the gentian violet, for the whole surface was now of the deep yellow color which this medium would have if the dye had not been added. Although at first restricted to the surface of the medium on which the colonies were growing, the decolorization extended deeper day by day until after ten days all but a small area at the back of the slant was decolorized. There was no liquefaction of the medium. On opening the tubes only a trace of the original color returned after exposure

⁹ J. Bact., 1924, 9, p. 211.

to the air for several days, showing that the dye was not merely decolorized by transformation into a leucobase, but was almost completely destroyed. The partial return of color was evidently due to diffusion of the unaffected dye, because subsequent cultures in which incubation was sufficiently prolonged to decolorize all the dye, failed to show any return of color after long exposure to the atmosphere.

The decolorization of gentian violet in Petroff's medium by certain organisms may prove to have considerable value in differentiation and identification. In the few tests made so far we have observed it in *B. sordellii*, *B. sporogenes*, and *B. novyi*; there was no trace of decolorization by *Bact. coli*, *Bact. typhosum*, or *Bact. aerogenes*, though all grow well on this medium. It is well known, of course, that *Mycobact. tuberculosis*, for which the medium was designed, does not markedly decolorize the dye. It may be that decolorization is merely a function of anaerobic growth, because some anaerobes that we have tested (e. g., *B. welchii*) failed to grow and so far none of the aerobic organisms of which growth was secured, decolorized the dye.

We tested the fermentation reactions of *B. sordellii* in constricted tubes¹⁰ containing beef heart infusion plus 2% Difco peptone, and 0.5% NaCl, made sugar-free by means of *B. welchii*¹¹ and adjusted to P_H 7.0. The carbohydrates were sterilized in 20% aqueous solution in the autoclave and added to the freshly boiled sugar-free medium just before inoculation from cultures in brain medium. Comparative tests were made with sugar solutions sterilized by filtration through Mandler filters but the results were identical with those in which the sugar solutions were sterilized in the autoclave. Acid production was taken as the principal criterion of fermentation and was tested by removing a small amount of culture from beneath the marble seal at 48 hours and adding a few drops of bromthymol blue in a porcelain test plate. Gas was formed also in these tubes, but was disregarded in the interpretation of the tests.

Our results showed that *B. sordellii* ferments glucose, levulose, and maltose, but not galactose, lactose, saccharose, salicin, mannitol, inulin, glycerol, or arabinose. Identical results were obtained with *B. sporogenes* in these tests. They also check exactly with the results reported by Sordelli, except that Sordelli left no record for salicin, or glycerol.

Pathogenicity for Laboratory Animals.—One strain of *B. sordellii* was highly pathogenic for guinea-pigs.

¹⁰ Univ. of Calif. Pub. in Pathology, 1915, 2, p. 147.

¹¹ J. Infect. Dis., 1921, 29, p. 344.

One animal, weight 630 grams, inoculated subcutaneously with 2 cc. of a 24-hour culture in glucose broth developed overnight a massive subcutaneous edema, with marked muscular weakness and prostration, and died in less than 24 hours. Necropsy showed the subcutaneous fascia markedly edematous, without hemorrhage or gas, just as in animals killed by *B. novyi*. Only a few gram-positive rods could be stained from the edematous tissues; these were mostly single but a few were in pairs. The viscera were normal, cultures of heart blood were sterile, and smears from the surface of the liver showed no organisms.

Further tests showed the above to be a characteristic result, except that generally no bacteria could be stained at the site of inoculation. Doses of 1 or 2 cc. of 24-hour glucose broth cultures always killed large guinea-pigs within 48 hours, usually within 24 hours; smaller animals weighing 240-250 gm. were repeatedly killed with doses as small as 0.1 cc. But doses of 1 or 2 cc. fed by pipet had no effect.

In some cases there was in addition to the subcutaneous edema, congestion of the lungs and a collection of fluid in the pleural cavity and in some cases the peritoneum and pleura were thickened and showed areas of gelatinous infiltration. There was occasionally a slight amount of localized hemorrhage and emphysema, but generally not. The heart was always stopped in diastole. Heart blood cultures were always attempted and were always negative. Indeed it was often impossible to culture the organisms from the subcutaneous tissues.

These observations were made repeatedly, not only with the parent culture but also with the repurified daughter cultures. All of the tests pointed to the probability of a soluble toxin as claimed by Sordelli and we have confirmed its presence in glucose broth containing ground meat as follows:

Three 100 cc. flasks containing this medium were incubated both for 15 and for 40 hours. They were then filtered through small Mandler filters and tested for sterility by inoculating 5 cc. into deep brain medium and incubating for two days. No toxicity tests were made until these sterility tests proved sterile.

It is unnecessary to give the results in detail as they corroborated Sordelli's claims in every respect. The 15-hour filtrates were slightly less toxic than the 40-hour filtrates; 1 cc. of the former injected subcutaneously into small guinea-pigs reproduced the peculiar lesions of the whole cultures and produced death in two days, but similar doses of the latter produced death overnight (about 12 hours). Doses of 0.1 cc. of the 15-hour filtrates produced non-fatal edema in three days but the animals recovered; in the case of the 40-hour filtrates the animals died on the third and fourth days.

These filtrates were stored at 35-40 F. from August 1926 until May 1927, and again tested. There was some loss in potency, because, while 1 cc. doses of the 15-hour filtrates produced a localized subcutaneous edema all the animals recovered, but the guinea-pigs inoculated with equal doses of the 40-hour filtrates died in 1, 2, and 3 days respectively.

Several guinea-pigs were inoculated with cultures of the other strain but none died. However, these animals showed a slight subcutaneous edema and we consider this strain an attenuated form of the same species. There was never any resemblance of the lesions produced by either strain to the foul watery bleb sometimes produced by large

doses of *B. sporogenes*. This strain produced only the opaque type of colony in deep agar cultures; its morphologic, tinctorial, and cultural characters were essentially identical with those of the pathogenic strain.

Sordelli^{2c} was unable to protect guinea-pigs against his new organism by mixtures of antiserums for *B. welchii* (*perfringens*), *B. septicus* (*Vibrio septique*), *B. novyi* (*B. oedematis*), and *B. histolyticus*, or by serums prepared against Sacquepee's *B. bellonensis*, and *B. sporogenes*. He later¹² advocated the use of polyvalent serums in the treatment of anaerobic gangrenous infections, following the practice of Weinberg and Sequin,¹³ but including an effective anti-toxin against his own organism. We have, as yet, done no serological work with *B. sordellii*. Such studies should be undertaken soon, in addition to morphologic and cultural comparison of *B. sordellii* with the new pathogenic anaerobe just described in a preliminary paper by Meleney, Humphreys, and Carp¹⁴ which seems to be closely related and may prove to be identical.

SUMMARY

This paper confirms Sordelli's discovery of a new putrefactive pathogenic anaerobe in human gangrenous infections, which combines the virulent properties of *B. novyi* with the principal morphologic and cultural properties of *B. sporogenes*.

The binomial "*Bacillus sordellii*" is suggested as a more appropriate and less confusing name than "*Bacillus oedematis sporogenes*" which Sordelli used.

Efforts to show that Sordelli's culture consisted of a mixture of different species were unsuccessful. It was possible, however, to isolate from one of his strains two substrains differing from each other in the form of colonies in deep agar, which are regarded as mutants or dissociates. Colonial differences, though at first apparently constant, were later shown to be temporary.

B. sordellii differs from *B. sporogenes* in pathogenicity, in motility, and in its slower and less complete proteolytic powers and from *B. novyi* in its more active proteolytic powers. It differs from both in the ability to form or accumulate tyrosine crystals in old protein cultures. It closely resembles *B. novyi* in the production of a soluble exotoxin and in the character of lesions produced in animals.

¹² Rev. Asoc. med. Argentina, 1923, 36, p. 223.

¹³ La Gangrene Gazeuse, 1917; Compt. rend. Soc. de Biol., 1923, 89, p. 463.

¹⁴ Proc. Soc. Exper. Biol. & Med., 1927, 24, p. 675.

A COMPARISON OF REACTIONS TO DERMOVACCINE AND TO NEUROVACCINE FOR SMALLPOX

STANLEY THOMAS

From the Laboratory of Bacteriology, Lehigh University, Bethlehem, Pa.

Statistical evidence demonstrating the efficacy of vaccine virus in protecting against smallpox is so overwhelming that no one today can intelligently contradict it. The dangers involved in its use, however, cannot be denied. Its preparation, while conducted with the utmost care and under the best scientific supervision does not preclude the chance of serious consequence due to contaminating bacteria. The staphylococcus present in the ordinary commercial vaccine virus (in spite of the glycerol and phenol preservative) may not be in itself pathogenic but by aiding anaerobic conditions it certainly can, and does, prepare a favorable field for the growth and toxin development of the tetanus bacillus. Although not in the vaccine itself, this organism is an opportunist in any wound, and tetanus following vaccination is always to be feared. To prepare a vaccine, therefore, which is entirely germ free and yet potent is the hope of all sanitarians. Various methods have been suggested for freeing vaccine virus from associated bacteria.

Fornet's ether method destroys the virus. Kirstein's¹ use of "eucupinotoxin hydrochloride" has been commented on favorably by Krumbach.² Iller³ used a concentrated solution of neutral trypanflavine and claimed excellent results. Groth and Arnold⁴ tested the relative value of chinol, trypanflavin, phenol and eucupinotoxin as preservatives of cowpox vaccine. The disinfectants all showed power to sterilize the lymph quickly but also reduced the virulence of the vaccine, and it was concluded by these workers that glycerol still remains the best preservative.

Park uses a 1:10,000 solution of brilliant green in cleaning the vaccinated area of the calf before removal of the pulp. This cuts down the number of contaminating organisms to a marked degree.

The cultivation of vaccine virus in vitro also has been attempted but without uniform success although Parker and Nye⁵ claim that it can be cultivated in

Received for publication, June 11, 1927.

⁵ Am. J. Path., 1925, 1, p. 325.

¹ Deutsch. med. Wchnschr., 1919, 42, p. 1102.

² Ztschr. f. Immunitätsforsch. u. exper. Therap., 1922, 34, p. 477.

³ Deutsch. med. Wchnschr., 1922, 48, p. 227.

⁴ Ibid., p. 1580.

tissue cultures composed of normal tissue; the presence of previously infected, living cells not being necessary.

Immunization with vaccine which has been heated so that the contaminating organisms are killed has been attempted by Groth,⁶ Morata⁷ and others but without success.

Noguchi⁸ between the years 1915 and 1918 proposed the use of a germ-free virus obtained by passage through the testicles of rabbits and bulls. Noguchi's testicular vaccine seemed for a time the fulfillment of the hope of the users of vaccines. A potent bacteria-free virus was obtained and was available for use. Experience soon showed, however, that virus prepared in this way rapidly lost its potency and was therefore unreliable.

The idea of using the brain of living rabbits as the culture medium for growing vaccine virus was an outgrowth of a note to the Societe de Biologie by P. Marie in the early part of 1921. Marie reported that he had succeeded in accustoming the vaccinal virus to rabbit brain and had produced fatal results in a few days.

In May of the same year Levaditi, Harvier and Nicolau⁹ started an investigation attempting the verification of Marie's statements, with the result that they were able to obtain a fixed virus adapted to the brain of rabbits. The method used by these workers was the alternate inoculation of virus into the testicles and brain of rabbits until a virus was obtained which would produce death by passage from brain to brain without alternating with the testicles. Later they claimed that this virus, which they called neurovaccine, could keep its virulence for nine months solely by cerebral passages. It retained its activity in glycerol in the ice box for at least 205 days.¹⁰ The virus produced an encephalitis in the rabbits on intracerebral inoculation but no crossed immunity could be demonstrated between the virus of vaccinia and that of epidemic encephalitis.

Levaditi¹¹ also showed that variola virus, while specifically different, apparently belongs to the encephalitis group but not to the rabies or poliomyelitis groups. In a resume of his work up to that time Levaditi¹² published a monograph in which he further stated that the neurovirus did not lose its affinity for the skin and could cause vaccinia in man.¹³ He¹⁴ also demonstrated that animals infected with vaccine virus cutaneously or in the cornea became immune after 16 to 25 days against intracerebral inoculation with this virus.

During the following year Camus¹⁵ in a critical review of Levaditi's work objected to the neurovaccine on two grounds: first, that it was not as efficient in producing vaccinias as ordinary vaccine virus grown on calves; second, from the type of reactions obtained it is probable that the neurovaccine is a mixture of a low grade vaccine virus and another virus which is a modified vaccine virus or an entirely different virus. Brunet and Conseil¹⁶ claimed that this vaccine causes hemorrhagic pustules on the skin of the inoculated rabbit (a property first described by Levaditi), as well as edema and necrosis. In man it frequently delays the pustulation and causes atypical pustules.

⁶ Ztschr. f. Immunitätsforsch. u. exper. Therap., 1923, 36, p. 534.

⁷ Ibid., 1924, 40, pp. 278-295.

⁸ J. Exper. Med., 1915, 21, p. 339; 1918, 27, p. 425.

⁹ Compt. rend. Soc. de biol., 1921, 85, p. 345.

¹⁰ Levaditi, C., and Nicolau, S.: Compt. rend. Soc. de biol., 1922, 86, p. 525.

¹¹ Ibid., 1921, 85, p. 425.

¹² Affinity of Vaccine Virus for the Central Nervous System, Monograph of the Pasteur Institute, 1922.

¹³ Levaditi, C., and Nicolau, S.: Compt. rend. Soc. de biol., 1922, 86, p. 77.

¹⁴ Ibid., p. 233.

¹⁵ Bull. acad. de med., 1923, 90, p. 79.

¹⁶ Compt. rend. Soc. de Biol., 1924, 90, p. 1408.

In what amounts to a reply to these statements Nicolau¹⁷ in the same year called attention to the report of work done by Levaditi and himself in 1923 in which they noted that the pustules produced on rabbits were different in appearance from those produced by dermovaccine. He observed pustules which were "frankly neurovaccinal, namely hemorrhagic, turgescient and necrotic."

In another paper Levaditi and Nicolau¹⁸ controvert the specific criticisms of Camus against the inferiority and harmful possibilities of neurovaccine. They present statistical evidence of the use of neurovaccine by Gallardo in the vaccination of 30,000 persons. Among these 96% of the primary vaccinations were satisfactory. They also present reasons (not convincing) for the appearance of atypical pustules and claim that it is not necessary to assume a symbiosis of two entirely different viruses.

This specificity for the virus was endorsed by Condrea,¹⁹ who also maintained that the cutaneous and testicular viruses have the same virulence for the nervous tissue, brain and spinal cord. In a study of the pathologic anatomy of the lesions induced by cerebral vaccine, the same author²⁰ showed that neurovaccine maintains its activity by brain passage for at least 9 months, and in glycerol for at least 205 days. Moreover it does not lose its neurotropic affinity by cutaneous passage.

The results of the work of Gallardo at the Institute of Alfonso XIII in Madrid were published in 1925. Gallardo²¹ concluded that 30 cerebral passages are necessary to produce a neurovirus of equal strength to the dermovaccine but that once obtained, neurovaccine is fully as strong as the vaccines in use and have the decided advantages of purity and availability. His procedure was as follows:

"A rabbit was inoculated in the testicle with virus which had already been previously subjected to testicular passage. The testicle was removed with the usual precaution and placed in a petri dish. We then divided it into three parts, the larger part was placed in a tube containing glycerine, second portion (smaller) was preserved in a tube of salt solution and the third piece (small) was placed in an ordinary bouillon tube. The latter was incubated at 37° C. We then placed on the ice the tube of saline solution and after having vigorously ground it we filled a syringe for the first intra-cerebral injection. The technique is very simple. After we had shaved the fronto-parietal region, having previously tied the animal, we disinfected the skin with tincture of iodine and made a slight linear incision through the periosteum; and applied a trephine in the median line to make a small round hole in the bone. Through this small orifice in the head we penetrated with a needle into the cerebral mass and gently injected 0.2 cm. of the saline testicular emulsion."

"Our first rabbit showed symptoms of paralysis on the third day; paralysis, jerking, difficult breathing on the 4th day; and died on the 5th day. The total number of rabbit passages were 89 and death occurred on the 5th and 6th day."

He found that the maximum virulence of his neurovaccine was not obtained with less than 30 cerebral passages. After 34 passages the vaccinal qualities were fully equal to those of dermovaccines.

The virus used in our experiments was obtained from Dr. Eduardo Gallardo of the Alfonso XIII National Institute of Hygiene through the kindness of Dr. D. Gonzalo Palacios. In a letter accompanying the virus Gallardo says:

"I am sending to you various pieces of cerebrum in glycerine, and I advise that you immediately inoculate various rabbits, since in such a long voyage it might again become attenuated. If all of the inoculated rabbits (at least 6) die from the fourth to the fifth day, you may be certain that the virus arrived in good condition, and after three or four additional brain passages, you can proceed to make whatever trials you wish. If one of the inoculated rabbits

¹⁷ Nicolau, S., et Poincloux, P.: *Compt. rend. Soc. de biol.*, 1924, 91, p. 1239.

¹⁸ *Bull. med.*, 1924, 38, p. 1441.

¹⁹ *Compt. rend. Soc. de biol.*, 1922, 86, p. 897.

²⁰ *Ibid.*, 1922, 86, p. 899.

²¹ *Ann. de l'Inst. Pasteur*, 1925, 39, p. 543.

does not die in the above mentioned time, it is advisable that you make passages from the brain to the testicle and from this to the brain (2 or 3 passages), repeating the brain passage up to 8 or 10.

"For trying the vaccination in man, make lots of 6 or more rabbits triturating the brains and emulsifying them in $\frac{1}{3}$ their weight of neutral glycerin diluted to 80% in sterile normal Saline Solution. The rabbits for inoculation must be healthy and young (3 to 4 months), it being absolutely essential to maintain them in quarters entirely apart from inoculated rabbits since they very readily become immune.

"For the first inoculation break carefully with the usual precautions the tube which I am sending. Pour out the glycerol and some of the pieces of brain tissue. Wash these several times with sterile salt solution and afterwards shake them hard in a tube containing 6 or 8 cc. of salt solution until obtaining an emulsion which is not very concentrated. With this emulsion, you will inoculate with a fine needle from $\frac{2}{10}$ to $\frac{3}{10}$ of a cc. If one of the animals should make quick movements of the head at the time of the inoculation, destroy it.

"All of the operations mentioned above must, of course be made aseptically. It is advisable to plant a few drops of the emulsion in ordinary bouillon, so as to make certain that the virus is not contaminated."

The history ²² of the lot of neurovaccine used is as follows:

Immediately upon receipt from Dr. Gallardo the virus labelled B -op.F was sent to the Hygienic Laboratory, U. S. Public Health Service, for test. On June 19, 1926, it was returned as being free from foot and mouth disease. After aerobic and anaerobic tests proved its sterility it was injected (0.2 cc. salt solution emulsion) into the brain of a rabbit. This rabbit died in five days (June 30). The brain was removed and the bulk placed in 40% glycerol. A small piece was shaken up with glass beads in physiologic salt solution and injected on July 2 into the testicles of a rabbit. This rabbit was killed on July 7. Almost complete engorgement of the testicles was found. The testicles were ground up with 4 times their weight of 40% glycerol. All aerobic and anaerobic tests were negative. On July 30th this preparation was injected into the brain of a rabbit. The rabbit was killed on August 3d and the brain removed. The bulk was placed in 40% glycerol and a small piece for inoculation was shaken in sterile physiologic salt solution. Bacteriologic tests showed the material sterile. Activity tests on a rabbit (skin test) showed a good vaccination. On August 12 this brain preparation was injected into the brain of a rabbit. On the 18th the animal was paralyzed and was killed. Necropsy showed slight meningeal congestion. Bacteriologic tests showed the material satisfactory. The next day, August 19, this virus (one passage through testicle and 3 through brain) was injected into the brain of a rabbit. The animal died on the sixth day (Aug. 25). Meninges showed no congestion although there was a slight discoloration of the cortex. The brain was removed into 40% glycerol. Aerobic and anaerobic plate cultures were sterile. On September 10, 5.3 grams of brain were emulsified and 25 cc. of 40% glycerol added. Eight aerobic plates were planted. Two of these showed one colony each, and the rest were sterile. Anaerobic tube cultures showed no growth, and safety tests in guinea-pigs showed the material innocuous. Activity tests on the skin of rabbits showed excellent results, although these tests on calves were not quite so good. The material was placed into tubes and submitted on September 13 to me. It must be emphasized at this point that the virus received from Gallardo was tested for potency on only one rabbit (instead of six) but that it died on the fifth day. There were four brain passages and one testicular passage before the virus was used.

One hundred tubes of the vaccine were used on human subjects on September 14, 1926. The technic used was the "pressure" method as described by Leake.²³ At the same time 351 vaccinations were made with a dermovaccine virus (table 1).

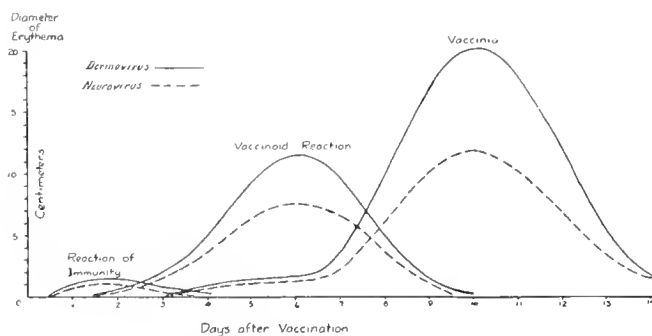
²² This work was done by Dr. W. F. Elgin of the H. K. Mulford & Co. Laboratories.

²³ Public Health Reports, 1927, 42, p. 221.

From this table it would be difficult to draw any unfavorable conclusions regarding the neurovaccine. From our past experience, however, with records of 1,527 vaccinations among men with the same general vaccination histories the percentage of immune reactions should have been considerably lower (about 62%) and the vaccinoids and vaccinia higher (about 25% and 12% respectively). In other words in the light of past experience both the neurovaccine and the ordinary vaccine used were of low potency. Among the men that had never been previously vaccinated (primary vaccinations) both of these lots of vaccine produced

TABLE 1
SUMMARY OF REACTIONS OF NEUROVACCINE AND DERMOVACCINE

	Neurovaccine		Dermovaccine	
	Number	%	Number	%
Immune reactions.....	84	84.0	271	77.2
Vaccinoid reactions.....	14	14.0	64	18.2
Vaccinias.....	2	2.0	16	4.6
Total.....	100	100.0	351	100.0



Comparison of local reactions to dermovaccine and neurovaccine.

vaccinias in every case. Of five men who had been vaccinated in the past but who showed no scar of the previous vaccination and were vaccinated with neurovaccine, two (40%) showed immune reactions, two (40%) showed vaccinoid reactions, and one (20%) had a vaccinia.

As to the type of reaction of the neurovaccine not a single one could be called hemorrhagic although the same virus produced hemorrhagic pustules on rabbits. On the contrary the reactions all showed a smaller extent of erythema than was usually seen with the dermovaccine. In the case of the vaccinia the extent of redness surrounding the vesicles was surprisingly small. In fact the vaccinia looked very much like the classical "pearl on a rose petal" without the rose petal.

Leake ²⁴ in his recent paper showed by diagram the reactions following vaccination. Using Leake's graphic method the accompanying diagram shows our results with nearly 2000 dermovaccinations and 100 neurovaccinations. It will be noticed that the reactions obtained by the neurovaccine showed a smaller degree of erythema than those obtained with dermovaccine.

Five months later 61 of the men vaccinated with neurovaccine were revaccinated with a fresh lot of dermovaccine of extremely high potency. The results shown in the table indicate the protection which the neurovaccine established against this dermovaccine.

TABLE 2
RESULTS OF REVACCINATION WITH DERMOVACCINE ON MEN PREVIOUSLY VACCINATED
WITH NEUROVACCINE

Number Persons Tested	Previous Vaccination (Neurovaccine)		Revaccination (Dermovaccine)	
	Reaction	Day of Reaction	Reaction	Day of Reaction
6.....	Immune	1	Immune	1
3.....	Immune	1	Immune	2
2.....	Immune	1	Immune	3
2.....	Immune	1	Vaccinoid	..
2.....	Immune	1	Vaccinia	..
7.....	Immune	2	Immune	1
5.....	Immune	2	Immune	2
7.....	Immune	2	Immune	3
8.....	Immune	2	Vaccinoid	..
1.....	Immune	2	Vaccinia	..
2.....	Immune	3	Immune	1
1.....	Immune	3	Immune	2
1.....	Immune	3	Immune	3
6.....	Immune	3	Vaccinoid	..
0.....	Immune	3	Vaccinia	..
2.....	Vaccinoid	..	Immune	1
1.....	Vaccinoid	..	Immune	2
2.....	Vaccinoid	..	Immune	3
1.....	Vaccinoid	..	Vaccinoid	..
0.....	Vaccinoid	..	Vaccinia	..
0.....	Vaccinia	..	Immune	1
0.....	Vaccinia	..	Immune	2
1.....	Vaccinia	..	Immune	3
1.....	Vaccinia	..	Vaccinoid	..
0.....	Vaccinia	..	Vaccinia	..

An analysis of this table shows that of 15 men who on vaccination with neurovirus had immune reactions on the first day (apparently indicating a high degree of immunity), 6, or 40% showed the same reaction on revaccination with dermovaccine five months later, while 9, or 60%, showed a lower degree of immunity. Three (20%) of the men who showed a high immunity to neurovaccine showed no immunity to the dermovaccine, as the latter produced vaccinas. Of 28 men who showed immune reactions in 2 days to neurovirus, 12 (43%) showed an equal or higher immunity later with dermovirus, but 16 (57%) showed less resistance and again one (3.6%) had not sufficient immunity to prevent a vaccinia. Of 10 men who had a slight immune reaction (3 day), 4 (40%) showed equal or better immunity on revaccination, and 6 (60%) again showed less. Of 6 men on whom neurovirus produced a vaccinoid reaction, all

²⁴ Public Health Reports, 1927, 42, p. 226.

except one showed an immune reaction against revaccination. One, however, again had a vaccinoid reaction. Finally (and this is rather significant) of the two men who had typical vaccinias with neurovaccine, one showed a slight (3 day) immunity and the other practically none against a dermovaccine only five months later. Individual susceptibility may have been solely responsible for this result as we only had two cases. But in the light of the other results it is noteworthy.

Let us compare the above results with a similar table showing the reactions obtained when both the first (i.e. in this study) vaccination and the revaccinations were made with dermovirus.

In summarizing this table we note that of those having immune reactions in one day, 83% remained immune at 24 hours on subsequent revaccination, while 17% showed a lower immunity on revaccination. Of those having immune reactions at 48 hours, 67% showed reactions 24 hours after revaccination, indicating a

TABLE 3
RESULTS OF REVACCINATION WITH DERMOVACCINE ON MEN PREVIOUSLY VACCINATED WITH DERMOVACCINE

Number Persons Tested	Previous Vaccination (Dermovirus)		Revaccination (Dermovirus)	
	Reaction	Day of Reaction	Reaction	Day of Reaction
29.....	Immune	1	Immune	1
3.....	Immune	1	Immune	2
3.....	Immune	1	Immune	3
47.....	Immune	2	Immune	1
19.....	Immune	2	Immune	2
4.....	Immune	2	Immune	3
18.....	Immune	3	Immune	1
6.....	Immune	3	Immune	2
0.....	Immune	3	Immune	3
12.....	Vaccinoid	..	Immune	1
4.....	Vaccinoid	..	Immune	2
4.....	Vaccinoid	..	Immune	3

higher immunity due to the first vaccination, 27% showed the same reaction on the second as on the first vaccination, and 6% showed a lower degree of immunity as represented by a third day immune reaction. Of those who on the first vaccination showed an immune reaction on the third day, 75% showed an immune reaction 24 hours after revaccination, and 25% showed an immune reaction in 48 hours. All of them showed some increase in immunity due to the first vaccination. Of those having vaccinoid reactions on the first vaccination, 60% showed immune reactions in 24 hours, 20% in 48 hours, and 20% in 72 hours, on revaccination. Again all showed an increase in immunity.

This table certainly indicates that immunity against vaccine virus can be increased without typical Jennerian vaccinia. In other words a revaccination with potent virus increases immunity whether it results in a vaccinoid or immune reaction. This fact is especially significant here as the original vaccination was made with a low potency virus. The difference between this table and table 2 is striking.

At the same time that these men were revaccinated for determining the degree of immunity, a new lot of men (103) who had not been vac-

inated by us previously were vaccinated with the same lot of virus. For comparison and for the sake of clarity the results of these vaccinations will be compared only with the revaccinations in those previously treated with neurovaccine.

Of the twelve men who had not been previously vaccinated by us and who got vaccinia, five had never been vaccinated before. As the results are compared with men who had been previously vaccinated these should have been left out of the comparison. If these five are deducted, the number of men reactive to the dermovaccine will be seven, or 6.8% of those not vaccinated within the past three years as compared with 5.0% of the neurovaccinated men. With this change the two tables are practically alike and it apparently made no difference in the results whether a man had been vaccinated with neurovaccine within five months or whether he had not been vaccinated within three years.

TABLE 4

REACTIONS TO SINGLE LOT OF DERMOVACCINE IN MEN PREVIOUSLY VACCINATED WITH NEUROVACCINE, AND IN MEN NOT VACCINATED WITHIN 3 YEARS

Type of Reaction	Revaccinated Men Five Months After Neurovaccination		Men Not Vaccinated Within 3 Years	
	Number	%	Number	%
Immune.....	40	66.0	60	58.4
Vaccinoid.....	18	29.0	31	30.0
Vaccinia.....	3	5.0	12	11.6
Total.....	61	100.0	103	100.0

There is no doubt that the neurovirus used in this preliminary work was of low potency. Whether this low potency was due to insufficient animal passage of the particular lot or whether it is inherent in a vaccine prepared in this way can only be shown by further experiments. Moreover it will be necessary to determine the keeping qualities of neurovaccine.

Recently it has been observed in Holland, Switzerland and elsewhere that the incidence of poliomyelitis and encephalitis apparently increased following vaccination against smallpox. In the light of Levaditi's work showing the similarity between the virus of vaccinia and that of encephalitis, and as some doubt as to the safety of the neurovaccine has been suggested by Camus, Brunet and others, considerable skepticism would probably have to be overcome by its users. Proof of the safety of any product rests with its proponents. In the recent past syphilis has received considerable attention as a possible congener of vaccination. Papers by Winkler, Kolb, Krapelin and Plant, and others have contro-

verted this theory and the same line of arguments may be used in defence of neurovaccine if it should prove to be an efficacious prophylactic against smallpox.

SUMMARY

In the one hundred cases studied, all reactions—immune, vaccinoid, and vaccinal—obtained with neurovaccine, were milder than the corresponding reactions with the ordinary dermovaccine. In no case did a hemorrhagic pustule result, and in every instance of pustulated accelerated reaction or vaccinia a clean, hard, non-itching scab formed quickly with almost no surrounding erythema. On revaccination with a high potency dermovirus, men vaccinated 5 months before with neurovirus did not show so high a degree of protection against the dermovirus as men who had previously (5 months) been vaccinated with dermovirus. These men did, however, show considerably more immunity than men who had not been vaccinated within three years. Definite conclusions regarding the efficacy of neurovaccine do not yet seem justified.

THE OPTOCHIN-FASTNESS OF PNEUMOCOCCI

CLAUS W. JUNGEBLUT

From the Division of Laboratories and Research, New York State Department of Health, Albany

Ever since that active and productive period during which Ehrlich discovered the arsenic-fastness of trypanosomes, the processes involved in the adaptation of microorganisms to various drugs and toxic substances have been studied from several angles. In addition to clarifying the biological significance of variability in microorganisms, it may reasonably be expected that research into the behavior of bacteria and protozoa under unfavorable environmental conditions will lead to a deeper insight into the changes that take place in the course of infection and immunity. Our conception of the phenomena of bacterial adaptation so far has been formed mainly from an accumulation of diverse observations on the morphology as well as on the cultural and serological characteristics of the adapted organisms. Scarcely any attempt has been made to explain the acquired resistance on the basis of a rational hypothesis (Pringsheim¹). This conception, however, has been considerably broadened in recent years by the contributions of Schnabel² and his collaborators.³ The results obtained by these authors seem to indicate that contact with the same substance (a disinfectant or a chemotherapeutic agent) may induce under certain conditions, even in the same experiment, specific fastness as well as specific hypersensitivity in various bacteria (pneumococcus, *B. coli*, *V. cholerae*). While it was found that bacteria grown in comparatively strong dilutions of a germicidal substance exhibited in the next passage a specifically increased tolerance for the same substance, those cultivated in weak dilutions of the same agent proved to be specifically hypersensitive under the same conditions. Between these two extremes there was generally found an inert zone which left the susceptibility of the bacteria unaltered for the next passage.

Received for publication, June 20, 1927.

¹ Die Variabilität niederer Organismen, 1910.

² Deutsch. med. Wchnschr., 1922, 48, p. 654; Ztschr. f. Hyg. u. Infektionskrankh., 1922, 96, p. 351.

³ Jungeblut, C. W.: Klin. Wchnschr., 1923, 2, p. 549; Ztschr. f. Hyg. u. Infektionskrankh., 1923, 99, p. 254.

The results of the *in vitro* experiments mentioned above appear not only to be supported but their value seems to be materially augmented by the later work of Schnabel and Kasarnowsky in conjunction with the author.⁴ These experiments demonstrated that, under similar conditions, pneumococci exhibiting specific hypersensitiveness to optochin may be recovered from mice treated with this drug after an experimental pneumococcus infection.

The fact that the reaction of the unicellular organisms to "foreign" substances may exhibit two phases, one of increased and one of lowered susceptibility, together with the strict specificity observed in these phenomena, suggests in general a certain resemblance between bacterial fastness and hypersensitiveness on the one hand and the processes of immunity and anaphylaxis on the other. However, while antigenic properties in the immunity reactions of the more highly organized animals belong, as a rule, only to high molecular substances of complex nature, the phenomena of specific fastness and of hypersensitiveness in bacteria may be elicited with crystalloids of simple chemical composition, such as sublimate and optochin (Schnabel).

One step further in our knowledge concerning the mechanism of bacterial fastness was made in 1924, when Schnabel⁵ reported on the successful transfer of optochin-fastness of pneumococci and sublimate-fastness of *B. coli* either by growing the nonadapted strains in the sterile filtrates of the respective fast cultures or by adding small amounts of such filtrates to the nonadapted strains while they were exposed to concentrations of the respective germicides, which inhibited the growth of control cultures without filtrate.

One phase of the present paper, publication of which has been delayed for over three years, deals with this same subject. It has been the aim in this study to undertake an investigation into the optochin-fastness of pneumococci with the object of examining certain cellular changes of the fast strain, as well as properties of the culture fluid, which may assume prominence in the establishment of this adaptation.

Experimental Work.—Since pneumococci become quickly adapted to optochin (ethylhydrocupreine) and since many different aspects of the optochin-fastness of pneumococci have been studied by earlier investigators,⁶ the pneumococcus and optochin were chosen for this study.

⁴ Klin. Wchnschr., 1923, 2, p. 682.

⁵ Ibid., 1924, 3, p. 566.

⁶ Morgenroth, J., and Kaufman, M.: Ztschr. f. Immunitätsforsch. u. exper. Therap., 1912, 15, p. 610; Tugendreich, J., and Rosso, C.: Ibid., 1913, 19, p. 156; Köhne, W.: Ibid., 1914, 20, p. 531; Moore, H. F., and Chesney, A. M.: Arch. Int. Med., 1917, 10, p. 611.

The strain used was a standard pneumococcus culture, type 1 (Neufeld), used in this laboratory for routine and research work; the optochin was the hydrochloric soluble salt of the drug (Numoquin hydrochlor. -Merck). The medium employed was a beef infusion broth, P_H 7.6; in a number of experiments it was enriched by the addition of 10% normal horse serum. By daily transfer of the pneumococcus strain, after incubation for 24 hours at 37 C., from one series of optochin-broth dilutions into another, the culture showing growth in the strongest concentration being always selected as the seed culture for the next passage, it was easily possible within a short time to achieve a high degree of adaptation of the pneumococcus culture to the substance used to induce fastness. A tenfold adaptation resulted after from 30 to 50 passages which could not be materially increased by further transfers. While the nonadapted strain grew on the average in optochin-broth dilutions of from 1:200,000 to 1:100,000 the adapted strain showed good growth in optochin-broth dilutions ranging between 1:10,000 and 1:20,000. Simultaneously, a control culture of pneumococci was incubated and transferred daily into broth without optochin. The two cultures obtained in this way were designated as the "fast" strain and the "normal" strain. The cultures were kept in semisolid serum agar in the cold room and used as parent cultures for the experimental work.

The two strains were practically alike as regards their morphology, growth on blood plates, bile solubility, and inulin fermentation. The specificity of the acquired tolerance and its persistence after prolonged cultivation of the organisms in optochin-free medium were found to be as marked as described in earlier papers.³ The virulence of the two strains was studied with the following results: of two rabbits injected intravenously with 0.05 cc. of the normal and of the fast strain, respectively, the one receiving the normal strain died after less than twenty hours while the fast strain caused death only after almost six days. There was practically no difference in the virulence of the two strains for mice.

Before the properties of the culture fluid of the fast strain were examined, some basic characteristics of the fast pneumococcus cell were studied. First, the number of living cells in the different cultures was determined in dilutions of "fast" and "normal" broth cultures with salt solution, by mixing 1 cc. of a 1:1,000,000 dilution with serum agar, and pouring plates. After incubation for 24 hours about the same number of colonies of both strains had developed. Then, the cellular metabolism was studied by means of the methylene-blue reduction, which is a very sensitive indicator of biological changes, provided the number of organisms is kept constant and controlled. In several tests it appeared that the normal pneumococcus strain reduced the methylene blue more quickly and completely than did the adapted strain.

Since adsorption may represent a preliminary phase in the process of disinfection, it seemed important to secure more data concerning the adsorption of optochin to the cells of the fast and normal strains and also to compare the adsorption of this drug on the surface of the streptococcus cell for purposes of control. The following experiment was therefore devised:

Fifty cc. of broth inoculated with pneumococcus "normal," pneumococcus "fast," and hemolytic streptococcus, respectively, and grown for 24 hours at 37 C. were centrifugalized and the sediment was suspended in salt solution (approximately 3 cc. in each instance) so as to make suspensions of equal density. Four different mixtures were prepared by combining 2 cc. of an optochin dilution of 1:10,000 in each case with 2 cc. of the bacterial suspension mentioned and with 2 cc. of salt solution as a control, which resulted in an optochin concentration of 1:20,000 in these mixtures.

TABLE 1
GROWTH OF NONADAPTED PNEUMOCOCCUS TYPE 1 STRAIN IN BROTH WITH THE ADDITION OF VARYING AMOUNTS OF SUPERNATANT FLUID FROM MIXTURES OF BACTERIAL SUSPENSIONS AND OPTOCHIN DILUTION

Amounts of Supernatant Fluids Added to 2.5 Cc. of Broth	1 Optochin + Salt Solution, Hours		2 Optochin + Pneumococcus Fast, Hours		3 Optochin + Pneumococcus Normal, Hours		4 Optochin + Streptococcus, Hours		Final Concentration of Optochin
	24	48	24	48	24	48	24	48	
	Cc.								
0.1	++	++	++	++	++	++	++	++	1:520,000
0.2	0	+	+	++	±	++	++	++	1:270,000
0.4	0	0	0	++	0	++	0	++	1:145,000
0.6, 0.8, and 1.0	0	0	0	0	0	0	0	0	1:103,333; 1:82,500, and 1:70,000

Control tubes of the normal pneumococcus strains grown in broth showed luxuriant growth after 24 hours.

These mixtures were held at room temperature for two hours, centrifugalized, and the supernatant fluid of each was tested for its inhibiting effect on the growth of normal pneumococci by adding increasing amounts to 2.5 cc. of broth. All tubes were then inoculated with 0.05 cc. of pneumococcus normal culture and incubated, growth being determined after 24- and 48-hour periods. The results obtained are given in table 1.

As may be seen from table 1, it appears that under the conditions of the test it was possible to remove about half of the germicidal power of optochin by adsorption to the pneumococcus cells; that the degree of this adsorption with the adapted and the normal strain was the same; and that under similar experimental conditions, the streptococcus cell adsorbed just as much optochin as the pneumococcus cell. Recent studies of Miller⁷ indicate that optochin is likewise adsorbed to the meningococcus cell.

⁷ J. Immunol., 1926, 12, p. 467.

Studies of cellular changes of the fast culture having revealed no characteristic changes associated with the process of adaptation, the culture fluid was examined for properties that might play a part in the establishment of this phenomenon.

The hydrogen-ion concentration of fast and normal cultures grown in broth and serum broth (cultures of different ages and also their filtrates were tested) was determined repeatedly, since it had been shown by Michaelis and Dernby⁸ and Ishimori⁹ that the reaction of the medium plays an important part in the activity of the antibacterial properties of the cinchona alkaloids. There was practically no difference between the P_H of the fast and of the normal cultures and of their respective filtrates under varying experimental conditions, the values obtained generally running between P_H 7.0 and P_H 6.8.

It was further thought interesting to study conditions of oxidation in the two cultures in view of the fact that Avery and Morgan,¹⁰ basing their work on the earlier observations of McLeod and Gordon,¹¹ had been able to demonstrate that the pneumococcus culture under certain conditions of growth produces H_2O_2 detectable by the benzidin-peroxydase test. By following the technic described in detail by these authors, it was found that the normal culture in broth and in serum broth produced, as a rule, more H_2O_2 than the fast culture in the same medium. In further tests it was shown that there was no relation between H_2O_2 in small amounts and the pneumococcal properties of weak optochin solutions.

Finally, experiments were carried out to determine whether or not optochin-fastness could be transmitted to a pneumococcus strain of normal tolerance by adding small amounts of the sterile filtrate of the fast strain to the normal strain while the latter was exposed to concentrations of the drug which would inhibit the growth of the normal strain without this filtrate.

The fast and, as a control, the normal pneumococcus cultures were grown in uniform amounts, 30 cc., of either plain broth or serum broth at 37 C. for different periods, which included the phase of active growth and the phase of autolysis, that is, 10 hours, 24 hours, 48 hours, and seven days. Furthermore, conditions of aeration were varied in both series by growing cultures aerobically and under partial exclusion of

⁸ Ztschr. f. Immunitätsforsch. u. exper. Therap., 1922, 34, p. 194.

⁹ Ztschr. f. Hyg. u. Infektionskrankh., 1924, 102, p. 323.

¹⁰ J. Exper. Med., 1924, 39, p. 275.

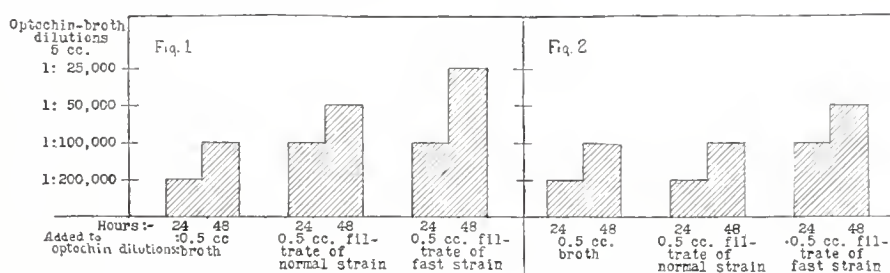
¹¹ J. Path. & Bact., 1922, 25, p. 139.

air, by covering them with a layer of sterile mineral oil. Filtration was carried out by means of small Berkefeld or Mandler filters. The filtrates were tested for sterility by aerobic, and occasionally also by anaerobic tests.

The technical procedure used most extensively for testing the capability of the filtrates to transmit fastness was as follows: three series of optochin concentrations covering the known range of growth and somewhat stronger concentrations (1:200,000 to 1:20,000) were prepared with plain broth. Each tube contained a total volume of fluid of approximately 5 cc. To each tube of one series a definite amount (from 1 cc. to 0.1 cc.) of filtrate from the fast strain was added, while to each tube of the second series an equal amount of filtrate from the nonadapted culture was added, as a control. The last series of optochin dilutions contained instead similar amounts of broth. All the tubes were then inoculated with 0.05 cc. of an 18-hour broth culture of the normal pneumococcus strain and observed for growth after different intervals of incubation, such as 8, 12, 18 and 24 hours, and two and three days. Generally, no further changes occurred after incubation for 48 hours, so that, with a few exceptions, this reading was taken as final. The purity of the culture in the tubes that showed growth was verified by microscopical examination.

In all, 23 filtrates obtained from the fast culture were tested for their capacity to transmit the fastness of the strain from which they were derived to a normal pneumococcus culture. Of these 23 filtrates, 13 were from cultures grown in serum broth and 10 from cultures grown in broth without the serum. Five of these filtrates were obtained from anaerobic cultures; the remaining 18 filtrates from aerobic cultures. In two experiments, another normal pneumococcus strain was selected for testing the effect of the filtrate from the fast culture; i. e., the pneumococcus, type 1, received in 1921 from the U. S. Hygienic Laboratory, which was a strain much more sensitive to the bactericidal action of the drug than was the pneumococcus culture used for the major part of this study. When evaluating the observations made with these 23 filtrates, growth occurring in the series of optochin dilutions was considered as due to transmission only when in the control tubes, containing either filtrate from a normal culture or broth instead of filtrate, there was no evidence of growth in the corresponding optochin concentrations. With only 7 out of the 23 filtrates tested was there definite evidence of transmission of fastness, 14 entirely failed to show transmission, in two cases growth was more rapid during the first 24 hours

than in the control cultures, but at the time of the final reading this difference had disappeared. In the seven instances in which transmission was indicated, it was obtained with five 24-hour filtrates, both from aerobic and from anaerobic cultures, with one 48-hour filtrate and the filtrate of a 7-day culture. A higher percentage of what was considered to be transmission of fastness occurred with filtrates secured from serum-broth cultures (4 of 10) than with filtrates from cultures in broth without serum (3 of 13). The extent of transfer of fastness varied widely from experiment to experiment. As a rule, it was such that, in the presence of the filtrate from the fast culture, growth of the nonadapted strain ensued in double the optochin concentration that was observed in the control tubes (figs. 1 and 2). Heating the filtrate of the fast strain for one-half hour at 60 C. did not affect its activity in two



Figs. 1 and 2.—Growth of nonadapted pneumococcus strain in optochin-broth dilutions with the addition of filtrates from the normal and fast strains.

experiments. There was no uniform quantitative relation between the degree of the induced higher tolerance to optochin, and the amount of filtrate added.

The specificity of the increased optochin tolerance transmitted to the normal cultures by means of filtrates from the fast culture, was tested with two filtrates which had previously been shown to be capable of inducing growth of the normal strain in double the inhibiting optochin concentration. It was found in both instances that the addition of 0.5 cc. of "fast filtrate" to various tr cresol dilutions was not followed by any growth of the normal pneumococcus strain in a higher concentration of tr cresol than what occurred with 0.5 cc. of broth instead.

In no case was the increased tolerance transmitted to the normal culture by contact with the filtrate from fast cultures, demonstrable beyond the first passage; in subsequent tests such cultures exhibited invariably their original tolerance for optochin. It will be noted from figure 1 that occasionally the filtrate from the normal strain likewise

induced a higher optochin tolerance in the normal strain for the next passage. This fact deserves special attention since Schnabel in his previously mentioned paper⁵ apparently was unaware of it. Obviously, this control is indispensable in evaluating the results.

When summing up the results, it is apparent that under carefully controlled technic, in a certain small percentage of cases, a relative, yet considerable, degree of temporary specific optochin-fastness may be observed in normal pneumococcus cultures by growing them in optochin-broth dilutions to which have been added small amounts of sterile filtrates obtained from a fast culture. Varying the experimental conditions in the manner described failed to disclose any factors of primary importance for the establishment of this transmission. The phenomenon is inconstant in character, since at present it has not been possible, arbitrarily, to duplicate results in repeated tests even by conscientiously adhering to the same technical details. It seems improbable, however, that factors, such as medium, conditions of growth, age of culture, play more than an accidental part in this problem, because transmission was seen with both serum-broth and plain broth filtrates, with filtrates secured from cultures grown aerobically and anaerobically, with filtrates of cultures of all ages, both the original strain 5 and another type 1 pneumococcus strain (U. S. Hygienic Laboratory) being used as indicators.

DISCUSSION

The fact that, in the presence of sterile filtrates from a fast pneumococcus culture, growth of a nonadapted pneumococcus strain may occur in otherwise inhibiting dilutions of optochin, may be interpreted as due to biological changes of the bacterial cells under the influence of the filtrate from the fast culture, or as the result of a possible destruction of optochin. The former assumption would seem at first sight to be strengthened by the fact that in a few cases it has been possible to induce a higher optochin-tolerance for the next passage in a normal pneumococcus strain which had been cultivated for 24 hours at 37 C. in the sterile filtrate of a fast culture. However, when the centrifugalized bacterial cells of the normal culture grown in the filtrate from a fast strain in broth were washed before being transferred into the series of optochin-broth dilutions no increase of the optochin-tolerance was observed. This fact seems to indicate that by transferring such a culture without this precaution small amounts of filtrate were transferred simultaneously, which may act in a manner discussed fur-

ther below. In another experiment which seems to preclude to a certain extent a change of normal pneumococci under the influence of the filtrate from a fast culture, the normal strain was cultivated in a sterile collodion bag surrounded by broth inoculated with the fast strain. After cultivation under these conditions for 24 and 48 hours in order that the possible influence of dialyzable substances of the fast culture on the normal strain might be studied, it was found that the tolerance of the latter for optochin had remained unaltered. Finally, the fact that in every case when fastness was transmitted it was lost in the next passage is a strong argument against the assumption that the filtrate may act on the bacterial cells. This quick disappearance of passively acquired increased tolerance contrasts sharply to the tenacity with which actively acquired adaptation is retained by the microorganisms.

The observations recorded in this paper on the higher optochin-tolerance of normal pneumococci, passively acquired in the presence of sterile filtrates from a fast culture, may in the writer's opinion more adequately be interpreted as suggesting the elaboration of specific soluble substances in the culture fluid of the fast strain capable of destroying the pneumococidal properties of the drug. The fact that occasionally the filtrate of a normal strain manifested similar properties, although less marked, would not invalidate the supposed mechanism in the action of the filtrate from the fast culture since the immunity reactions of the more highly organized animals differ likewise only quantitatively and not qualitatively from the normal physiologic reaction. The significance of such a conception for the mechanism of optochin fastness of pneumococcus is obvious. In the absence of any reliable chemical test for the quantitative determination of optochin in high dilutions, it is impossible to furnish conclusive proof for the outlined hypothesis. Again, while Voegtlin, Dyer, and Miller¹² recently advanced a working hypothesis for an explanation of the arsenic resistance of trypanosomes on a chemical base, such a way of approach cannot be adopted for the problem of optochin-fastness in pneumococci, since nothing definite is yet known of the chemical interaction between quinine and its derivatives and the protoplasma of the pneumococcus cell.

In this connection, however, it is of interest to record the observations of Neuschlosz,¹³ who studied the mechanism which appears to be responsible for the acquired resistance of *Paramecium caudatum* against quinine and certain dyestuffs. He has been able to show (1920)¹³

¹² J. Pharmacol. & Exper. Therap., 1924, 23, p. 55.

¹³ Arch. f. d. ges. Physiol., 1919, 176, p. 223; 1920, 178, p. 61.

by quantitative chemical analysis, that paramecia which are fast against quinine have acquired the property of destroying the drug, while normal paramecia lack it almost entirely. In a later study, he succeeded in demonstrating by colorimetical methods that the fast organisms had acquired the property of destroying the dyestuff employed to induce this fastness. In 1923, the writer³ described a staphylococcus strain which was adapted to methylene blue and which was distinguished from the original strain by its quicker reduction of the dye. Neuschlosz concludes that fastness in both instances may be due to the secretion of defense ferments ("Abwehrfermente").

CONCLUSIONS

Studies of cellular changes of a pneumococcus type 1 strain adapted to optochin, did not disclose any characteristic properties which could be attributed to the adaptive process.

A nonadapted pneumococcus type 1 strain in the presence of sterile filtrates of an optochin-fast variety of the same strain may occasionally exhibit a specifically increased tolerance for optochin in the next passage.

Optochin-fastness manifested by a nonadapted pneumococcus strain in the presence of filtrate from a fast pneumococcus strain does not seem to be associated with biologic changes in the nonadapted pneumococcus cell.

BACTERIA CONCERNED IN THE SPOILAGE OF HADDOCK

2. DISSOCIATION OF AN ORGANISM RESEMBLING *B. VULGATUS*

A. H. GEE*

From the Laboratory of General Bacteriology, Yale University, New Haven, Connecticut

In a preliminary report on microorganisms isolated from haddock of the Atlantic coast, the author¹ presented evidence indicating that gram-positive rods which predominate in the flora of these fish constitute a single individual species in the subtilis-mesentericus group which is capable of existing in at least two distinctly different modifications. One of these, characterized as the "active" form, was recovered regularly from the muscle of fish which had become stale as a result of storage for one week. It produced uniform turbidity in broth and grew on agar plates as gray, circular colonies possessing a glistening surface. This form, which was assigned a share in the mechanism of spoilage, passed after subculture and storage of several weeks in the laboratory into an "inactive" modification which gave surface scum in broth without apparent growth below, and developed as dry, wrinkled, scalloped colonies on agar.

Transfers from the slime and muscle of living haddock revealed a predominating type having precisely the same characteristics as the "inactive" or rough variant which was derived by continued culture from the smooth strains previously obtained from stale muscle. The hypothesis was advanced that the two forms are an example in the subtilis-mesentericus group of the rough-and-smooth type of bacterial variation observed by numerous investigators of other groups of bacteria. This phenomenon has been styled "microbic dissociation" by Hadley² in a recent comprehensive review. Organisms susceptible to this S-R variation can split into two sharply defined forms which may differ culturally and morphologically, in antigenic power and in pathogenicity.

As a starting point for the observations in the present paper there had been built up a representative collection of strains which included

Received for publication, July 3, 1927.

* Fellow of the National Research Council.

¹ Contributions to Canadian Biology and Fisheries (Toronto), 1927, 3, p. 347.

² J. Infect. Dis., 1927, 40, p. 1.

both the "active" form of the organism encountered in the muscle of stale haddock, and the "inactive" form from the muscle and slime of the living fish. It was proposed to determine whether these bacilli are actually a single species, and if so to study the complementary S and R guises.

Before undertaking the description and comparison of the cultures, stock transfers were started from single cells, using the micropipet method according to the directions of Kahn.³ The manipulations were performed under the oil-immersion lens and the technic for preparing coverslip films was modified in order to make certain that both spores and vegetative cells in the drops under examination would be equally visible.

The isolations were made directly from agar slant cultures showing a high percentage of spores after incubation for several days at 30 C. Single spores were planted into broth and incubated at 37 C.; when growth took place it was usually visible on the following day. The successful growths numbered about 1 in every 10 transfers. Occasionally, growth developed after two or three days, but such strains were not included in the collection owing to the possibility of their having developed from weak and hence atypical spores.

Some 38 representative single-cell strains of the fish organism were preserved on agar slopes and kept at room temperature. The sources of the cultures were as follows.

From fresh fish:

Slime of live fish, 7 strains
Muscle of live fish, 10 strains
Slime of fresh fish at New York plant,
6 strains

From stale muscle:

Isolated August 1925, 10 strains
Isolated August 1926, 5 strains

Characteristics of Single-Cell Strains.—Nutrient mediums used in the study of the strains were adjusted to P_H 6.8, and all cultures with the exception of gelatin were incubated at 37 C.

Agar Slopes: The stock cultures of the organisms on nutrient agar slopes, were identical in appearance. The track of the loop used for inoculation was covered by a heavy, moist, opaque, creamy growth which did not spread much beyond the inoculated area. Folds or wrinkles did not develop and the moist appearance was retained for many weeks. The material could readily be removed from the surface of the medium, but it was not viscid.

Nutrient Broth: Surface scum developed after incubation for 1 day in every case. This broke into fragments and settled down when the cultures were shaken, and was replaced by a new surface film when the incubation was continued. All cultures showed some turbidity in the liquid in addition to the surface scum.

³ J. Infect. Dis., 1922, 31, p. 344.

Potato: When growing on potato plugs, the strains showed a general resemblance, with individual differences. In two days, the top of the stroked area bore a moist, yellowish or creamy growth spreading only slightly from the needle track. The lower area showed a brownish, spreading growth with fine wrinkles and a dry surface. After four days the whole surface of the plug was covered with a heavy mixed growth of moist cream patches and dry, finely wrinkled brown areas. In a week the potato plugs were blackened and covered on the sides as well as on the inoculated surface with a moist, clayey deposit showing wide ridges. It was not possible by making periodic transfers of the same strain on potato to duplicate the picture exactly, since the dry wrinkling would be more in evidence at some times and the moist yellowish deposit at others.

Agar Plates: When broth cultures were plated out on nutrient agar, it was evident at once that the surface colonies were of two strikingly different forms. The majority were dry, opaque and wrinkled with an irregular margin, and adhered to the medium. They could not be removed with a needle excepting in their entirety or in fragments. A small percentage of the colonies were bluish, translucent, glistening, circular and viscid. There were also occasional creamy, opaque colonies to which reference will be made later. From 1 to 10% of the colonies on each plate were smooth and the amount of S component did not appear to bear any relation to the source of the strain in question.

This indicated that the previously observed roughening of the "actives" or smooth cultures from stale muscle had again taken place; but in addition, the "inactives" or roughs from the living fish had displayed a trend in the reverse direction, and now contained some smooths. Thus each of the stock strains contained a mixture of organisms bearing either R or S characteristics, with a preponderance of those tending to R.

The original stock single-cell strains were inoculated into the usual differential mediums for the sake of comparing them prior to attempts to split off R and S substrains. The types displayed a striking similarity in behavior. Quantitative differences on certain of the mediums occurred which were set aside as without significance, since they were not reproduced in cultures in the same mediums in confirmatory tests.

Microscopic Appearance: Variations in size and shape of the "inactive" forms were noted at the commencement of the problem.¹ It was now found that as a result of the working-over to which the strains had been subjected before they were secured in single-cell culture, a degree of stabilization had taken place. In 24-hour cultures in plain broth or on nutrient agar, the organism was found as a rod with rounded ends from two to five times as long as wide, with average dimensions of 0.8μ by 2.5μ . The tendency to variation in size had not entirely disappeared, however, as most of the cultures contained many short, thick forms, as well as long, thin rods. All cultures were definitely gram-positive.

Spore Formation: Spores were formed readily on agar slants in the incubator. The spores survived the customary heat test at 80 C. for 10 minutes when suspended in nutrient broth. Mature spores were cylindrical and without end caps, and measured approximately 0.5μ by 1.0μ , again with some variation. Spores were formed centrally as a rule and without appreciable bulging of the sporangia.

Motility: The rods were actively motile in 24-hour cultures in liquid or on solid mediums, with tumbling or undulating movements. They could be observed to cruise rapidly around the edge of a small drop containing a single cell.

Gelatin Stabs: Nutrient gelatin was inoculated by stabbing with a straight needle, and held at 20 C. After one day, funnel-shaped liquefaction had commenced along the upper half of the stab. In four days, the growth was napiform with a heavy scum. The medium was liquefied as far as the walls of the tube

in one week, and subsequent liquefaction proceeded downwards in layers, with the scum persisting. Four to six weeks were necessary for complete liquefaction. At 37 C. complete liquefaction required a week.

Litmus Milk: The strains acted slowly on litmus milk and did not produce extensive changes in acidity. After incubation for 4 days, coagulation had commenced, leaving the medium clear just below the surface and faintly acid. The reaction then turned to alkaline and coagulation and digestion proceeded simultaneously, so that in two weeks there was a soft coagulum occupying the lower half of the volume of the medium, and the upper half had become a clear liquid, colored blue to brown. Complete digestion took place in three weeks, leaving the liquid dark brown or dirty blue.

Sugar Mediums: Bromthymol blue was added to nutrient broth containing the respective carbohydrates. Glucose and sucrose broths were turned completely acid in both arms of the fermentation tubes in from four to seven days, during which growth developed as turbidity and sediment, but without scum. Scum and sediment formation and clouding took place in lactose broth, but no acid was formed. In glycerol broth a friable scum was formed, with slight production of acid, after one week. No gas was produced in these four mediums, and the control tests showed that acidification when present was attributable to utilization of the carbohydrate in the medium.

Differentiation of S and R Forms.—Examination of smears made from different 48-hour colonies on agar plates inoculated from the stock single-cell strains revealed an important difference. The smooth colonies were composed entirely of vegetative cells, and the rough colonies consisted mainly of spores, with and without sporangia. The smooth colonies remained without spores despite further incubation. No growth resulted when the heat test was carried out on material from the smooth colonies suspended in nutrient broth. The rough colonies survived the heat test and on transfer produced a tough scum in broth, leaving the liquid clear below.

Cultural Differences: Both forms bred true during several successive transfers in broth and on agar alternately at 24-hour intervals. The roughs gave rough colonies, 100%, and scum in broth; the smooths produced smooth colonies, 100%, and only uniform turbidity in broth, without any surface scum (fig. 1).

The two forms were readily distinguished on potato slants. Plugs inoculated with smooth substrains produced a creamy to yellowish growth with a moist or glassy surface and spreading little from the line of inoculation. Rough substrains gave a growth showing a tendency to spread and with innumerable fine wrinkles, in appearance dry brown to powdery gray.

Nutrient agar slants containing 3% peptone also revealed a striking difference between the S and R forms. The roughs formed an opaque, wrinkled skin adhering firmly to the medium and colored light

fawn with brown stains; the smooths gave bluish-gray, translucent, viscid growth. Photographs were taken (fig. 2) of 48-hour growths of the R and S substrains on this medium, plated and inoculated with three needle strokes for each form.

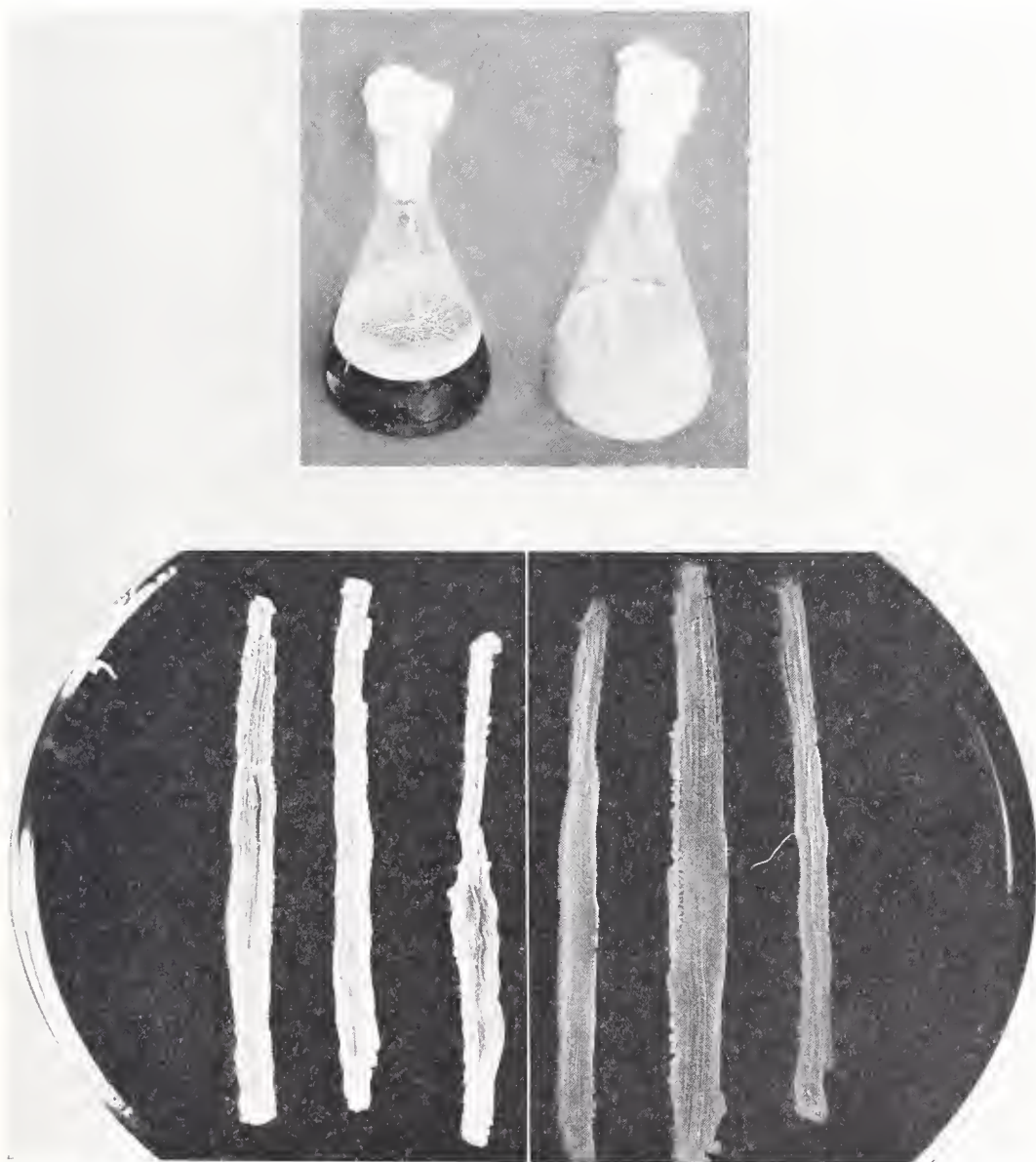


Fig. 1.—*B. "mesentericus-vulgatus"* in liquid medium (above): left, R form; right, S form, in nutrient broth 48 hours.

Figs. 2 and 3.—*B. "mesentericus-vulgatus"* on 3% peptone agar (below): left, R form, dull, opaque, fawn colored and adherent to medium; right, S form, glistening, translucent, bluish-gray and viscid.

Similarities in Behavior: The average dimensions cited for the rods held for both rough and smooth substrains. Both variants were gram-positive and actively motile. Differences could not be established in the rates at which milk, gelatin or carbohydrates were attacked.

Identification of Organism.—From their parallel action on the various culture mediums all strains, including the “inactives” obtained from living haddock and those derived from “actives” originally secured from stale muscle, were evidently a single species. The problem of identification was complicated by the wide differences between the two variants, especially in the appearances of the growths on potato, which is frequently relied upon as a medium for differentiating the members of the subtilis-mesentericus group. Further, it did not seem reasonable to select the rough form as the normal one and the smooth as an accidental variant, or vice versa.

The rough form of the haddock organism does bear a general resemblance to *Bacillus vulgatus* as catalogued in the elaborate description of aerobic spore-formers of Lawrence and Ford.⁴ Species from the collection of Doctor Ford with which he favored this laboratory were studied in conjunction with the fish strains. The reactions toward milk, gelatin and carbohydrates, the shape of mature spores, and the cultural appearances of the R form, linked the unknown closely with *B. vulgatus*. The properties of the smooth strains of the unknown, especially the appearance of the growths on potato, indicated, however, that there was at least some resemblance to *B. mesentericus* of Ford. Consequently the fish organism may best be known for purposes of reference as *Bacillus mesentericus-vulgatus*, retaining the old trinomial which identifies it as *vulgatus*, and at the same time covers the mesentericus tendencies.

Interconvertibility of Roughs and Smooths.—The presence of both R and S forms in single-cell strains obtained from cultures of which some formed rough colonies when secured and others produced smooth colonies, indicated a degree of interchangeability. It has been noted that, when once split off, the R and S substrains retain their characteristics for several passages in broth and on agar. It was of interest to study conditions which favor the transformation of spore-bearing substrains into asporogenous ones, or conversely, which restore spore formation in a strain which has become asporogenous.

⁴ J. Bact., 1916, 1, p. 273.

The appearances of colonies of the R and S substrains are exactly the same as those described for sporogenous and asporogenous *B. anthracis* by Rosenthal,⁵ who was able to develop asporogenous races by periodic transfer of a spore-bearing race in the filtrate (Berkefeld) of either an R or an S culture. Three such passages, with intervals of five days between them, could produce a completely asporogenous race. Making use of this analogy, a flask culture in nutrient broth of the R component of one of the stock strains was passed through a Berkefeld filter and dispensed in 5 cc. quantities into sterile culture tubes, with precautions against contamination. This medium was inoculated from the stock slope of the same R component used to prepare it, and then incubated for five days. The characteristic scum formation associated with R components never took place in this medium, and growth developed only as a uniform turbidity. Colonies were still rough, subcultures in plain broth formed a scum, and spores were still present. Successive transfers into the filtrate were made every five days, and at each transfer agar plates were poured, spore tests performed and nutrient broth tubes inoculated. The growth in the filtrate was invariably uniform and always without a film. After the third subculture in the R filtrate, a small proportion of smooth colonies appeared on the plates, and transfers to plain broth then failed to form a scum, although spores were still present. This indicated a progressive change, which was carried through to completion in the fifth subculture in the same filtrate medium. At the end of this treatment poured agar plates developed 100% smooth colonies, and heat tests for spores were negative, showing that the original R substrain had been converted into its completely asporogenous counterpart.

It has been noted that the reverse change, S to R, usually takes place slowly and spontaneously in stock cultures. The recovery of spore-formation by an S substrain was found to take place most quickly in liquid cultures of large volume. A 500 cc. flask containing 300 cc. of nutrient broth was inoculated with a 10 cc. broth culture of an S substrain and incubated. During the first four days the growth was uniform and no scum appeared. After further incubation a scum developed and in the course of two weeks this became so tenacious that it could not be broken up by shaking. Spores were demonstrated by the heat test. When plated out, spore-containing colonies were obtained which were not like either the characteristic roughs or smooths, but were

⁵ Compt. rend. Soc. de biol., 1926, 95, p. 445.

creamy and opaque. Thus although the reversion was only partial, the ability to form spores had been successfully restored.

Intermediate Forms.—The creamy colonies in the foregoing experiment were similar in appearance to occasional creamy colonies found when the stock single-cell strains were plated out, of which mention has been made previously. In color, texture and surface appearance they were also similar to the growth produced on agar slants by the stock strains. These colonies appear to occupy the place of the indefinite "Zwischenformen" discussed by Hadley² as intermediates between true R and S forms. At present it is not possible to decide whether these creamy colonies result from a mixture of the two types of cells, which separately tend to either S or R form, or whether they consist of uniform cells each of which is in process of changing its mode from the one form to the other.

DISCUSSION

Bearing on Microbic Dissociation.—The dissociation phenomenon described is further confirmation of the view that rough-smooth variation occurs in all groups of microorganisms. Apparently the first observation of variation in the subtilis-mesentericus group ranked definitely under this heading is the study of Soule⁶ on R and S forms of *B. subtilis* proper and the use of immune serums for interconverting them, to which reference is made by Hadley.² The present paper adds a second organism, and in addition emphasizes the question of spore-formation in relation to dissociation.

The data also link up closely with the experiments of Rosenthal⁵ on a third member of the group, *B. anthracis*. The colony characteristics of the sporogenous and asporogenous races of the two species, and the ease with which spore-formation can be suppressed, are exactly similar.

Taxonomic Significance.—The divergence in culture of the R and S forms of the organism reintroduces the question of classification in the subtilis-mesentericus group. Colony features and the nature of the growth on potato have been shown to differ sharply and widely in the case of the two dissociants of the haddock organism. It is consequently doubtful whether published descriptions of the less common members of the group can be strictly applied for purposes of classification, since wider acquaintance with these rarer species might reveal other characteristics, which become emphasized by dissociative changes, and which may link the numerous but uncommon individuals to better known

⁶ J. Infect. Dis., 1927, in press.

aerobic spore-formers. The experience with *B. "mesentericus-vulgatus"* shows that the appearance of growth on potato, or other essentially cultural characteristic, should not be considered invariable in describing and differentiating species in the *subtilis-mesentericus* group, but that physiologic activity should be taken into account also. This question is considered by Perlberger⁷ who has supplemented descriptions of *B. mycoides* and closely related species or types with a study of the fermentative reactions of the organisms.

It is possible that, failing a study of the entire group from the standpoint of dissociation, the older view of Gruner and Fraser⁸ might well be adopted. These authors make five subdivisions of the group.

The observations on the haddock bacillus, which possesses characteristics of both *B. vulgatus* and *B. mesentericus*, assume further significance in conjunction with the study of Haag⁹ who presents evidence which may be regarded as indicating that *B. mesentericus* and *B. megatherium* constitute a single species.

Relation to Fish Spoilage.—The S form derived by dissociation from "inactive" organisms isolated from the slime and muscle of live haddock cannot be distinguished from variants of "active" organisms present in stale muscle. The S forms of the "inactives" do differ morphologically from the "actives" proper, however, in one respect. The "actives" give lens-shaped subsurface colonies, while the subsurface colonies of the S forms of the "inactives" are irregular squares. Variations in single-cell cultures of "active" types of the organism from stale muscle are at present receiving attention, and if the attempt to reverse the whole process of degradation in culture tubes should prove unsuccessful, it is hoped that nevertheless it will be possible to follow the convergence of the two types to some intermediate stage, using single-cell strains throughout.

The absence of spore formation in the S forms of the "inactives" is of significance. It establishes a further bond with the types from stale muscle, since it has recently been found difficult to demonstrate spores in them by the heat test when they are freshly isolated. It is possible that further study of the haddock flora may permit the establishment of even wider identities among bacteria, because still other types, differing principally in their gram-staining reaction, can be secured from the muscle of fish kept for a period of 10 days. Of interest in this connection is the observation of Miss Newton,¹⁰ who,

⁷ Centralbl. f. Bakteriöl., 2, O., 1924, 62, p. 1.

⁸ J. Infect. Dis., 1912, 10, p. 210.

⁹ Centralbl. f. Bakteriöl., 2, O., 1926-7, 69, p. 4.

¹⁰ Contributions to Canadian Biology (Toronto), 1923, N. S. 1, p. 379.

during a study of marine spore-forming bacteria, recovered from haddock an organism which closely resembles *B. "mesentericus-vulgatus"* as described in this paper, and differs mainly in that it is reported gram-negative.

SUMMARY

An organism identified as *B. "mesentericus-vulgatus"* has been isolated in variant forms from the muscle of stale haddock and from the slime and muscle of live haddock.

This bacillus, which exists in asporogenous and sporogenous forms, constitutes an example of microbial dissociation in the *subtilis-mesentericus* group.

Data are presented which have a bearing on the problem of the classification of this group.

TRANSMISSIBLE LYSIS OF A THERMOPHILIC ORGANISM

STEWART A. KOSER

From the Hygienic Laboratory, University of Michigan, Ann Arbor, and the Department of Bacteriology, University of Illinois, Urbana

The list of bacteria which have been found to be susceptible to the lytic principle, or bacteriophage of d'Herelle, has been an ever lengthening one as additional species have been subjected to the test. In most of the work thus far the organisms employed have been the better known pathogenic or saprophytic types which develop best at about 37 C. or in a few cases at lower temperatures. It seemed of interest, therefore, to determine whether the same phenomenon would be evident in the group of thermophilic organisms which are capable of growth at high temperatures. In the present investigation a thermophilic bacillus was employed. It was found to be susceptible to the d'Herelle phenomenon, not only at 37 C., but at temperatures considerably above this point. A preliminary communication¹ of this has appeared and additional data will be presented here.

The culture selected for investigation was one of a stock collection of thermophiles maintained in this laboratory. It was isolated originally from milk.² No attempt has been made to completely describe or name the organism and it has been carried in stock under the designation T60. It is a gram-positive spore-forming rod. The size of the rods varies somewhat with the type of medium and with the age of the culture. In stains made from 24-hour agar slants one sees rather slender rods of medium length, about 3.0 to 5.0 microns long. In very young cultures the cells are somewhat larger than this. In some instances the rods occur singly while in others they are attached end-to-end to form chains. This characteristic seemed to be associated with the type of growth in nutrient broth and with the type of colony, and will be discussed later under the section on dissociation.

The temperature relations of the organism were rather striking. The optimum for growth appeared to be in the range from 45 to 52 C. and here the culture developed much more rapidly than at 37 C. At

Received for publication, July 22, 1927.

¹ Koser, S. A.: *Proc. Soc. Exper. Biol. & Med.*, 1926, 24, p. 109.

² Tanner, F. W., and Harding, H. G.: *Centralbl. f. Bakteriol.*, 2, 1926, 67, p. 330.

57 C. the growth was less rapid than at 52 or at 55 C., while at 59-60 C. the limit appeared to be reached and the organism was barely able to multiply. At 62 C. there was no evidence of growth. At 20 to 25 C. development was slow and two or three days were required for production of a moderate growth on agar slants. The limiting temperature for growth may vary somewhat with the character of the medium and the above statements apply to beef infusion peptone agar, P_H 7.0. In slightly alkaline medium, P_H 7.6, development was not quite so rapid as in neutral medium. This was noted especially at temperatures above the optimum where development was distinctly retarded.

A lytic principle active against this organism was obtained by incubation of a filtrate of sewage-polluted river water with small amounts of young culture in broth. Beef infusion peptone broth, P_H 7.6, was used for this purpose. The mixture of culture, broth, and water filtrate, after incubation overnight at 37 C., was filtered and the filtrate again brought in contact with the culture and incubated as before. This process of alternate "feeding" and filtration was repeated several times, always incubating at 37 C. since at this time it was unknown whether the bacteriolytic principle would be capable of activity at 50 C.

The lytic principle developed with surprising rapidity. The second filtrate showed some evidence of lytic power while the third gave a distinct clear streak when a small drop was allowed to run down the surface of an agar slant previously inoculated with the culture. After development at 37 C. the lytic filtrate was found to be very active when brought in contact with the T60 culture growing at 52 C. Both in broth and on agar lysis occurred more rapidly at the higher temperature and it is deemed a matter of interest that a gradual adaptation of the lytic principle to the higher temperature was unnecessary.

The "thermophilic" lytic principle fulfilled all of the tests commonly accepted for demonstration of the bacteriophage. It was filtrable, lysis was transmissible in series, clearing was produced on agar cultures and in appropriate dilutions lytic areas were formed. Before being used for further study it was carried through a series of broth cultures at 50 C. to such an extent that the last tube represented a dilution of 1×10^{-10} of the original sewage polluted water. This should insure the disappearance of any "heterologous" bacteriophagic or other properties which might have been contained in the original sewage filtrate.

Quantitative Measurement.—After the above serial passage through broth cultures the "concentration" of bacteriophage was tested. Dilu-

tions of a lytic filtrate ranging from 10^{-1} to 10^{-12} were made in 10 cc. amounts of broth and one drop (approximately 0.05 cc.) of a young broth culture added to each tube of the series. Readings were made after 3, 5, 17 and 24 hours at 50 C. Complete clearing of the culture occurred in 10^{-9} , while the 10^{-10} dilution showed growth of the culture comparable to that of control tubes. The inhibition titer of the lytic principle is therefore 1×10^{-9} . A repetition of the test yielded similar results. A test of lysis made by adding the dilutions of bacteriophage to 5-hour broth cultures which showed visible growth, also gave the same result and showed complete clearing in a dilution of 1×10^{-9} but not in higher dilutions.

Arcs.—The characteristics of the lytic areas were of some interest. Due to rapid growth of the organism areas could be discerned against a light background of culture after about 6 hours at 50 C. After 16 to 18 hours they stood out in sharp contrast to the surrounding heavy growth of culture (fig. 1). The areas were usually 2-3 mm. in diameter with a somewhat irregular edge. The central portion of the area appeared perfectly clear while near the border there was a narrow grayish zone of incomplete lysis. This could be seen to best advantage when the culture had reached its maximum growth. The areas gave the effect of a scooping out of the culture with the sloping sides constituting the zone of incomplete lysis and leading to the clear central portion. At 37 C. the areas were somewhat smaller than at 50 C. but presented much the same appearance. At 25 C., as a result of slow growth of the culture, from 40 to 48 hours were required before lytic areas were apparent. After 3 to 4 days at this temperature growth was moderately heavy, the areas stood out clearly, and in appearance and size closely resembled those formed at higher temperatures.

Lytic Activity at High Temperatures.—An effort was made to get an approximate idea of the rate of increase of phage "units" at different temperatures, especially the higher temperatures in the neighborhood of 50 C. and above.

For this purpose a lytic filtrate was titrated and diluted in sterile broth to such an extent that one standard loop of the last dilution gave well isolated areas when brought in contact with the culture on agar slants; 10 cc. quantities of this dilution were then run into a number of sterile test tubes and again tested.

A 5-hour growth of the T60 strain from an agar slant at 45 C. was next taken up in 3 cc. of sterile broth, well emulsified to break up the

larger clumps, and then 0.3 cc. of this broth suspension of young culture was added to each of the above tubes of diluted lytic principle.

After addition of the culture suspension, the several tubes were held at different temperatures and the increase in lytic units was followed by withdrawing, at intervals, a loop of the mixture and streaking it evenly over an agar slant just previously inoculated with young culture. These agar slants were then incubated at 45 C. for 16 to 24 hours and the number of lytic areas counted. Where necessary, dilutions were made in sterile broth in multiples of ten and a loopful of the dilution was used instead of the original tube. No difficulty was experienced in counting the areas of lysis. When made from appropriate dilutions, the areas were well isolated and sufficiently large to afford a striking contrast to the surrounding heavy growth of culture.

The results are given in table 1. Here it is seen that the original mixtures of diluted bacteriophage and culture contained from 16 to 39 phage units per loop. In the presence of the young culture the number of units rapidly increased until rather high dilutions were necessary to give well-isolated areas. The most rapid increase was at 50 C. and this coincides closely with the optimum temperature for growth of the T60 culture. At lower temperatures the increase in phage units proceeds at a more moderate pace, while at higher temperatures (above 54 C.) there is an abrupt decline. The results at 56 and 58 C. are interesting, for here there is no real increase in the number of lytic units. Indeed, at the latter temperature there is a marked decline in their numbers, if one may rely upon the absence of lytic areas as an indication of destruction, or at least inactivity, of the lytic principle. At the same time the culture was capable of multiplication at this temperature, as shown by a slight increase in turbidity as well as by the development in control tubes of broth containing the culture alone. Such tubes, inoculated originally with a loop of culture suspension, showed a light clouding of the medium after 9 hours' incubation and a moderately heavy growth upon further incubation. Although slower than at 50 C., growth was nevertheless evident upon repeated tests. It is interesting that in spite of growth of the culture at 56 and 58 C., lysis did not take place.

In another series of experiments tests for lytic activity were made by bringing together the undiluted lytic principle and the culture on agar slants. Agar slants were smeared with a small amount of young broth culture grown at 50 C. and then a loopful of undiluted filtrate was allowed to run down the center of the slant. A number of slants with culture alone were used as controls. Several of the test slants, together

with controls, were then held at the temperature desired. In each case the precaution was taken to incubate the slants for a short time before inoculation in order to bring them to the desired temperature.

At 50 C. growth of the culture was very rapid. Slants containing the lytic filtrate showed a cleared streak in the center where the filtrate had been applied and heavy growth at the sides of this zone. At 54 C. development of the culture was quite rapid and the slants to which the filtrate had been added presented much the same appearance as at 50 C. At 57 C. growth was considerably retarded, though the central clear zone of lysis was nevertheless evident. At 58 to 59 C. the culture was markedly retarded and instead of a uniform growth, small isolated colonies appeared on the slants. On those receiving the lytic principle there was no evidence of growth in the central part where the principle

TABLE 1
INCREASE IN NUMBER OF LYTIC UNITS AT DIFFERENT TEMPERATURES

Incubation Temperature, Centigrade	At Start	Number of Areas per Loop (Dilutions in Parentheses)		
		4 Hours	9 Hours	22 Hours
37	23	85 (10^{-1})	48 (10^{-4})	30 (10^{-5})
		5 (10^{-2})	4 (10^{-5})	5 (10^{-6})
47	34	16 (10^{-2})	32 (10^{-5})	57 (10^{-5}) 6 (10^{-6})
50	16	90 (10^{-3})	110 (10^{-5})	39 (10^{-5})
		8 (10^{-4})	14 (10^{-6})	5 (10^{-6})
54.5	39	10 (10^{-2})	17 (10^{-4})	61 (10^{-4})
			3 (10^{-5})	16 (10^{-5})
56	20	32	11	1
58	34	2 (10^{-1})	1 (10^{-1})	
		2	0	0 (also negative after 46 hours at 58 C.)

had been applied, while a number of small isolated colonies appeared at the sides of the slant. Control tubes showed similar colonies over the entire slant. In this case, while the result was not clear cut, there nevertheless appeared to be at least an inhibition of the culture by the lytic principle. At 60 C. growth was confined to a very few small colonies and was so scanty that conclusions could not be drawn from the result.

In performing tests of this kind to determine the maximum temperature for activity, there is evidence for believing that the potency of the lytic principle will influence the result. When a loop of undiluted lytic filtrate was used at a critical temperature, such as 56 or 58 C., a cleared zone could be detected against the background of culture on agar slants. When such a filtrate was diluted to give only a few areas per loop, and a loop of the dilution applied to an agar slant culture at the same tem-

perature and in a similar way, frequently no areas of lysis could be seen. Controls made from the same diluted material and held at lower temperatures—50, 45, or 37 C.—showed the few areas of lysis very nicely.

Other factors also appear to influence markedly the results at the highest temperatures: the composition of the medium—beef extract or beef infusion—slight changes in the hydrogen-ion concentration, and perhaps other unrecognized factors associated with the “dissociation” of the culture. It therefore appears impossible to state exactly the maximum temperature permitting activity of the T60 lytic principle. The critical zone, however, is about 56 to 59 C., with the exact result depending on the conditions of the test.

Inactivation by Heat.—Heat resistance tests were made in thin-walled glass tubing 3 mm. in diameter. After introduction of a lytic filtrate, the tubes were immersed in a water bath and held at the desired temperature for 30 minutes. Upon completion of the heating period they were cooled quickly and tests for activity were made at once. Inactivation of the lytic principle was determined both by streaking a loop of the heated material on each of two agar slants just previously inoculated with culture, and by the addition of 10 drops to a tube of sterile broth which was then inoculated with a small amount of young broth culture. In this way activity of the lytic principle after heating could be detected by lysis on agar and in broth.

Since the T60 lytic principle was active at temperatures fully 20 degrees or more above the optimum for most organisms, it was thought that it might be more heat resistant than the usual bacteriophage. Accordingly, preliminary tests were made at intervals of 5 degrees from 65 to 85 C. Here it was found that it survived 70 C. for 30 minutes but not 75 C.

Another experiment to determine more accurately the point of inactivation is given in table 2. Two lytic filtrates were used in this test. Both had been prepared by lysis of a young broth culture at 50 C., but they differed in the length of time allowed to elapse between the completion of lysis and the subsequent test. Filtrate 1 had been allowed to “age” by holding in the ice box for one week prior to use, while filtrate 2 was tested within 24 hours after lysis of the culture. An interesting difference is brought out in the resistance to heat of the old and young filtrates. Although containing approximately the same concentration of phage units per cc., as shown by titration, the older filtrate (1) is appreciably more resistant. In this case 74 C. evidently represents about the limit of heat tolerance, for one loopful streaked over an

agar slant showed no evidence of lysis in one instance, while in the other only two lytic plaques appeared. The fresh filtrate (2) was less resistant and was appreciably weakened at 72 C., while uniformly negative results were secured at 74 C. These results are in agreement with the previously work of d'Herelle³ and of Tomaselli⁴ with a Shiga bacteriophage.

It appears also that the bacteriophage "units" within the same suspension are not all inactivated at the same temperature. This has been demonstrated by De Necker⁵ and it is seen also in this case. One loop of the unheated phage always cleared an agar slant which had been smeared with the culture and high dilutions were necessary to obtain well isolated areas. Just before complete inactivation, however, the number of units is so reduced that only a few areas resulted upon application of a loop of the heated undiluted phage. Perhaps the most interesting point brought out by the heat resistance tests is the demon-

TABLE 2
INACTIVATION OF THE LYTIC PRINCIPLE BY HEAT

Temperature, C. 30 Minutes	Filtrate 1 Titer 1×10^{-9}			Filtrate 2 Titer 1×10^{-9}		
	1 Loop on Agar Slants		10 Drops in Broth	1 Loop on Agar Slants		10 Drops in Broth
70	+	+	+	+	+	+
72	+	+	+	+	+	+
74	0	+	+	0	0	0
76	0	0	0	0	0	0

+ = lysis on agar slant culture or clearing of broth culture; 0 = absence of lysis or clearing (normal growth of culture). Number of lytic areas indicated in parentheses.

stration that the thermophilic lytic principle is no more heat resistant than the usual coli or dysentery bacteriophage and is inactivated at about the same temperature.

Resistant or Secondary Growth.—The absence of resistant or secondary growth at ordinary temperatures was quite striking. After lysis at 50 C. by a bacteriophage of maximum potency the cleared culture tube was usually removed to room temperature, kept overnight and used to reinoculate another tube in series. In such a manner bacteriophagy could be continued through a long series of broth cultures without recourse to filtration between successive passages. Broth cultures which had been completely cleared at 37 or at 50 C. and then held at room temperature without filtration, showed no macroscopic evidence of

³ Compt. rend. Soc. de biol., 1920, 83, p. 274.

⁴ Quoted by d'Herelle: *The Bacteriophage and Its Behaviour*, 1926.

⁵ Compt. rend. Soc. de biol., 1922, 86, p. 736.

development of resistant organisms until after two or three weeks, and sometimes not then. Lysis of the culture on agar mediums under optimum conditions resulted in complete clearing of the area and secondary resistant colonies usually made a tardy appearance if the lysed cultures were removed from the 50 C. incubator and held at room temperature.

Continued incubation of lysed cultures at 50 C. resulted in a more speedy development of resistant cells and secondary growth usually appeared in from two to four days. In broth this frequently took the form of a diffuse clouding, in contrast to the normal culture which gave a decidedly granular or flaky growth. On agar the resistant colonies which appeared on the lysed areas varied in size and appearance. Some were small and convex with round even margins, while others were exceedingly irregular and frequently so thin and transparent as to be almost phantom-like in character.

At temperatures above the optimum, the lytic principle appeared to be weakened and secondary growth developed much more speedily. Thus in tests run at 54 C. after complete clearing of young broth cultures, secondary growth appeared in the form of a diffuse turbidity within 24 hours. In lysed areas on agar at the same temperature, secondary colonies developed readily and in some instances almost covered the area which had originally cleared. The secondary colonies at this temperature sometimes developed lytic centers, giving the colonies a ring appearance.

Dissociation.—The T60 culture was observed to undergo the phenomenon of "dissociation," as evidenced by the formation of rough and smooth colonies. The various factors which may bring about such a change are imperfectly understood, though it is evident from a recent review of the subject by Hadley⁶ that heat, food substances, immune serums and other conditions play a large part. Hadley's review should also serve to focus attention on the subject of dissociation in connection with studies of transmissible lysis.

During the present investigation the only factor studied was the temperature of incubation. The ordinary or "normal" culture, as kept in the stock collection, was usually grown at 45 to 50 C. and then after development it was stored at room temperature. Such a culture, on plating, consisted almost wholly of rough, dry-looking colonies with very irregular margins. Whether these represent the extreme R type

⁶ J. Infect. Dis., 1927, 40, p. 1.

is uncertain, for various degrees of "roughness" were observed from time to time (fig. 2). It was against such a culture that the bacteriophage was first developed.

By repeated transfers at room temperature it has been possible to develop an S type. In bringing about this change agar slant cultures were used and the first transfers were made at intervals of 2 or 3 days. Later, as the culture became better adapted to growth at the lower temperature, daily transfers could be made. The colonies shown in figure 3 resulted from plating the twentieth transplant. They presented a moist shining appearance and were of a soft butyrous consistency, in contrast to the dull dry tenacious growth of the original culture.

It was at first believed that the S type developed only at lower temperatures. Later, it was found that such was not the case, for by repeated quick transplants on agar at 50 C. it was possible to develop a strain in which smooth characteristics predominated. The colonies formed by such a culture, in their early stages of development, resembled closely those held at room temperature. The smooth appearance was only temporary, however, for on continued incubation for 24 hours or more at 50 C., irregular hair-like filaments developed at the margins of the colonies and they then began to assume more of the R characteristics. From various observations made throughout the course of this study, it appears that growth at high temperatures and a brief period of "ageing" of the culture are the chief factors responsible for the appearance of the R type. It should be noted, also, that various colony forms intermediate between those shown in figures 2 and 3 were also encountered from time to time. Undoubtedly, temperature is not the only factor which may bring about such changes. For example, it was noticed on several occasions during the repeated room temperature transplants that the small colonies developing in crowded areas of the plate showed a tendency to assume the intermediate or rough type of margin, whereas the well isolated colonies were smooth.

Attempts to retain the S type of growth in broth failed. Tubes of broth inoculated from a young agar culture of the S dissociate exhibited at first the uniform, even turbidity characteristic of smooth types. On microscopic examination at such a time the rods were found to occur singly, with very few pairs or chains. On further incubation, and usually within 24 hours, the growth changed to the granular or flocculent type. Subcultures made during the early stages of growth to another tube of broth likewise soon assumed the granular R character-

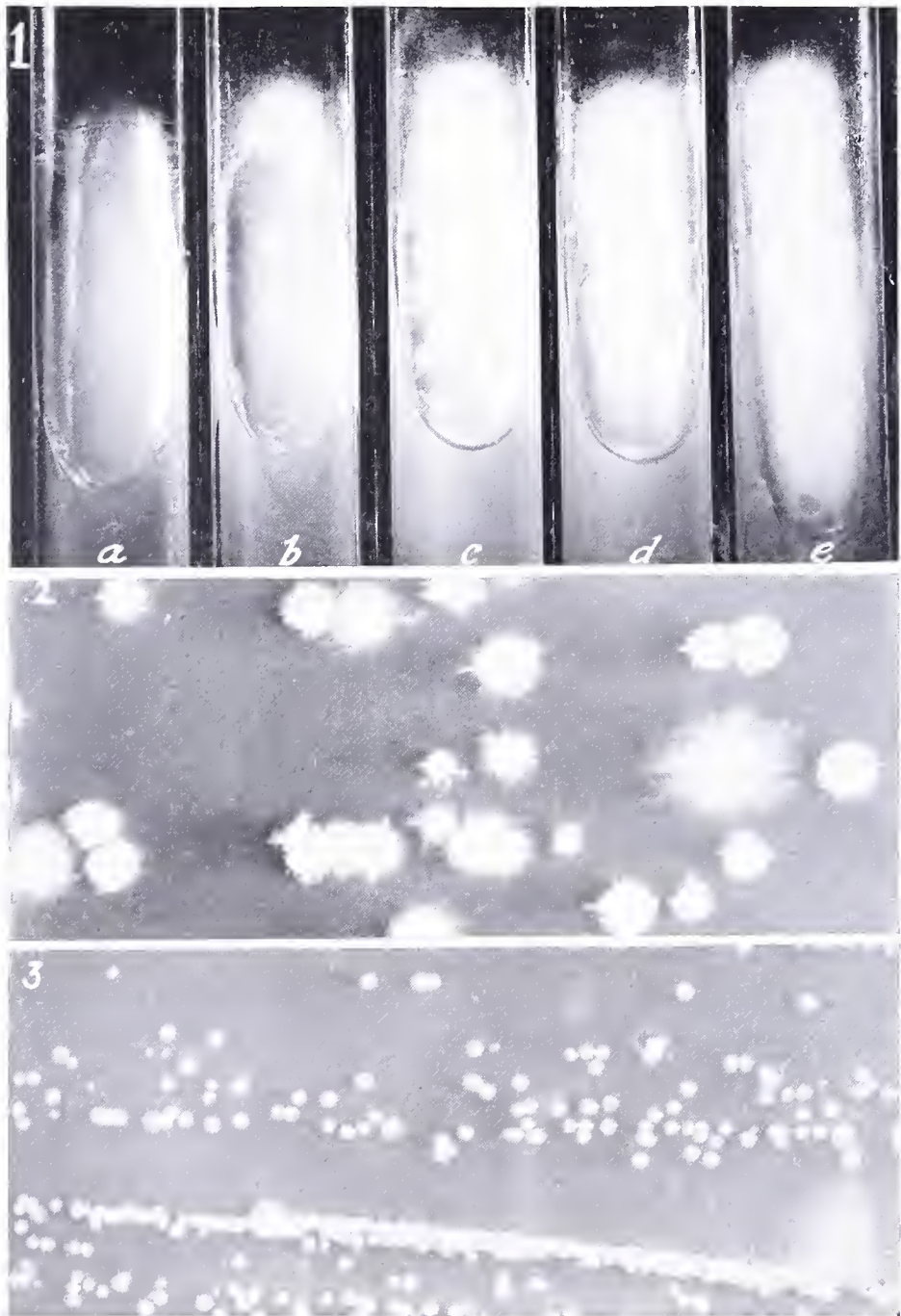


Fig. 1.—Lysis at 50 C. (a) Undiluted lytic filtrate applied to central portion of agar slant. (b to e) Successive dilutions of lytic filtrate applied to agar slant cultures, showing lytic areas.

Fig. 2.—Stock culture of thermophilic organism. Colonies on agar, two days at 45 C.

Fig. 3.—Thermophilic organism, showing type developed by twenty successive transplants at room temperature. Colonies three days at room temperature.

istics. Evidently the $S \rightarrow R$ change occurs very speedily in broth and the reverse process is difficult to bring about.

It appeared of interest to determine the action of the lytic principle against the S and R forms as represented in figures 2 and 3. The original lytic principle, before successive passages through broth cultures, had been preserved by sealing in glass and this was used in the first tests. It was equally active against both the S and R dissociates, as determined by either the number of lytic plaques on agar or by the inhibition titer in a liquid medium. The lytic principle which had undergone successive passages through broth cultures (R type) at 50 C. to such an extent that the final transfer represented a dilution of 1×10^{-40} of the original filtrate, was next used. In spite of its repeated lysis of the R type, it appeared to be equally active against the S culture which had been developed separately by repeated transfers at room temperature. In one aspect only was a difference noted and this was in the appearance of the lytic plaques on a solid medium. The areas of lysis in the S culture were round, with even margins, and lacked the outer zone of incomplete lysis which was so pronounced a feature with the R type of growth. Undoubtedly this difference can be referred, not to a change in the lytic agent, but to a change in the character of bacterial stratum. When the soft butyrous S type of growth was supplied, the areas resembled very much those formed by lytic agents against typical typhoid, dysentery or coli cultures. When the tenacious, dull, dry-appearing and irregular R dissociate was supplied, the areas were somewhat irregular in outline and the zone of incomplete lysis along the margin then became evident.

DISCUSSION

It is evident that the lytic agent which has been the subject of this study resembles the usual bacteriophage in most of its characteristics: the transmission through series of cultures, the formation of lytic areas, the titer as determined both by inhibition and by lysis, the point of inactivation by heat; all are similar in a general way to the numerous other lytic principles which have been reported.

The point of special interest to which this lytic principle seems entitled resides in its action at unusually high temperatures. With an optimum temperature centering about 50 C. and with the ability to produce lysis at temperatures as high as 57 to 58 C. the thermophilic bacteriophage is unique among those thus far described.

It is believed also that it may have some significance, though perhaps indirect, in connection with the discussion of the nature of the bacteriophage. If we accept d'Herelle's view that transmissible lysis is caused by a living organism, a distinct species of filtrable virus, we must assume the existence of certain members of this group which are capable of development at high temperatures. This of course is a possibility, although thermophilic filtrable viruses have not hitherto been described so far as the writer is aware. Since a thermophilic bacillus growing at high temperatures must carry on its metabolic activities and elaborate enzymes which function at high temperatures, it is evident that viewed from the standpoint of a metabolic or enzymic function, the phenomenon of transmissible lysis applied to a high temperature organism appears no more unusual than for other organisms. The phenomenon of lysis would seem to be something very close to the culture itself. Demonstration of the existence a high temperature lytic principle, then, seems to lend weight to the evidence against the conception of the bacteriophage as a distinct species of living organism.

SUMMARY

A transmissible lytic agent for a thermophilic gram-positive spore-forming organism was found to possess the unusual feature of activity at high temperatures (optimum about 50 C. and persisting to 57 or 58 C.) which suggests a close relation between the principle and the organism itself.

THE INCIDENCE OF SCARLET FEVER STREPTOCOCCI IN THROATS OF DIPHTHERIA PATIENTS

PAUL S. RHOADS

*From the Scarlet Fever Committee and the John McCormick Institute for Infectious Diseases,
Chicago*

Because of the lack of work on the presence of scarlet fever streptococci in throats of diphtheria patients the search for these organisms in diphtheria was undertaken. This search seemed desirable for a number of reasons. In spite of the most rigid precautions against cross infections scarlet fever sometimes develops in patients admitted to diphtheria wards. Councilman, Mallory and Pearce¹ in a study of 220 deaths from diphtheria, reported scarlet fever 34 times. It has long been known that streptococci could be cultured from the complicating lesions of diphtheria more often than any other microorganisms. Until methods for the recognition of scarlatinal streptococci were worked out it was, of course, impossible to know how many of the complications were due to scarlatinal infection.

Interest in the problem was stimulated also by the observation that occasionally in the Durand Hospital a diphtheria patient who reacted positively to the Dick test on admission, became negative before leaving the hospital without developing a recognized rash. At times such patients have desquamated. The question naturally arises whether or not these may have been diphtheria patients or diphtheria carriers who were at the same time suffering from throat infections due to scarlet fever streptococci; but without developing a rash, or having a rash so transient that it was not observed.

That scarlatinal throat infection may occur without eruption has long been suspected by clinicians. In 1921 Dick and Dick² by experimental inoculations showed that such infections do occur. They swabbed pure cultures of different strains of hemolytic streptococci obtained from the throats of early scarlet fever patients on the tonsils and pharynx of 30 volunteers, producing sore throat associated with fever and leukocytosis but no rash in seven. In 1923³ using the same strains with

Received for publication, July 30, 1927.

¹ Diphtheria, a study of the bacteriology and pathology in 220 fatal cases, 1901.

² J. Am. M. A., 1921, 77, p. 782.

³ Ibid., 1923, 81, p. 1166.

which they produced typical scarlet fever they produced severe angina, fever and leukocytosis without rash in two more persons.

Distribution of Scarlet Fever Streptococci in Other Conditions.—In 1925, Williams,⁴ using the toxin neutralization method described by Dick and Dick,⁵ identified 42.8% of hemolytic streptococci from excised tonsils as well as one strain from the sputum of a patient with bronchitis and one from an osteomyelitis lesion as scarlatinal strains. Stevens and Dochez,⁶ using the same method, found 5 scarlatinal strains in the 17 hemolytic streptococci tested. These strains came from the throats of nurses with acute pharyngitis during an outbreak of scarlet fever in the nursing group. With agglutination and agglutinin absorption methods their percentage of scarlatinal strains was slightly higher. Nicholls⁷ studied 21 strains from sources other than scarlet fever patients, most of them from patients with tonsillitis, pharyngitis or sinusitis. Using the toxin neutralization method she identified ten of these as scarlatinal strains.

Method of Study.—In this study the incidence of hemolytic streptococci in the throats or noses of 100 patients admitted with a diagnosis of diphtheria to Chicago hospitals for contagious disease was first determined. These hemolytic strains were then divided into scarlatinal or non-scarlatinal strains by the toxin neutralization method.

The material for throat cultures was obtained by rubbing a sterile cotton swab over the tonsils and pharynx and for nasal cultures by deep insertion of a swab into both nares. The swabs were then placed in tubes containing 0.5 cc. of plain sterile broth each. On returning to the laboratory one or two drops of the broth so inoculated were spread over sheep blood agar plates. The same day Dick tests were done to determine the susceptibility of the patients in question to scarlet fever. Next day the growth on the plates and the skin tests were studied. Only one set of cultures was made from each patient. The length of time the patients had been in the hospital was not taken into account, some being in the acute stage of the disease, others convalescent. All cultures were made in the summer and early fall.

Single colonies were transferred from all the plates on which hemolytic streptococci appeared to blood agar slants. The 24-hour growth from a single slant was used to inoculate 150 cc. of plain broth to which sheep blood (0.5%) had been added. After four days in the incubator at 37 C. smears were made from the broth cultures to determine purity and enough phenol added to make 0.5%. Twenty-four hours later the cultures were passed through plain filter paper, then through Berkefeld N filters. Before using for skin tests the diluted filtrates were passed also through W filters.

⁴ Am. J. Pub. Health, 1925, 15, p. 129.

⁵ J. Am. M. A., 1924, 82, p. 265; 1925, 84, p. 802. Dick, G. F., and Dick, G. H.: J. Am. M. A., 1925, 84, p. 802.

⁶ Ibid., 1926, 86, p. 1110.

⁷ J. Clin. Invest., 1926, 3, p. 411.

The filtrates were tested for their scarlatinal toxin content by making suitable dilutions with physiologic salt solution and using these for intradermal injections of 0.1 cc. each in persons shown to be susceptible to scarlet fever by the Dick test. The first dilution of a filtrate was 1:1000 or 1:500. When such dilutions failed to produce reactions lower ones were used until a dilution was reached with which an erythema of at least 1.0 cm. in one diameter developed in 24 hours. Thus a quantitative estimate of the activity of the filtrate of a given strain was afforded. If a filtrate diluted 1:10 failed to cause a positive reaction in at least one of two or more persons known to react to the standard Dick test, no toxin neutralization tests were made with it.

Having determined for a given strain the dilution at which a positive skin test was produced, mixtures were made so that 0.1 cc. contained the previously determined skin test dose of the filtrate and at least ten neutralizing doses of antitoxin. After 1½ hours in the incubator at 37 C., 0.1 cc. of this mixture was injected intradermally in persons susceptible to scarlet fever. At the same time 0.1 cc. of a standard Dick test toxin, 0.1 cc. of the unmodified dilution of the filtrate which had given a positive skin test previously, and 0.1 cc. of the unmodified antitoxin dilution used in the mixture, were injected also. The results were read 20 to 24 hours later, the diameters of any areas of erythema were recorded in millimeters and the color noted as faint, moderate or bright. When the scarlet fever antitoxin neutralized the ability of a filtrate to cause a skin reaction the filtrate was shown to contain scarlatinal toxin, and the strain of hemolytic streptococcus which produced the toxin was identified as a scarlatinal strain.

Results of Cultures and Toxin Neutralization Tests.—In 100 cases diagnosed diphtheria, hemolytic streptococci were encountered 29 times, 27 times in the throat, 6 times in both nose and throat and twice in the nose alone. Sixteen or 55.2% of these hemolytic streptococci were identified as scarlatinal strains.

The tests by which the scarlatinal strains were identified appear in table 1. Positive reactions with antitoxin alone were frequent. Such tests are omitted from the table because they have no bearing on the final result. There was much variation in the strength of the toxins produced by the different strains under identical conditions. The following outline of the 16 toxins shows that only one strain was a strong toxin producer.

Highest reacting dilution of toxin.....	1:1000	1:500	1:150	1:100	1:50	1:20	1:10
Number of toxins producing positive skin reactions	1	1	1	2	4	3	4

The ability of four filtrates to produce positive skin tests was not modified by the addition of scarlet fever antitoxin (table 2). The streptococci producing these were therefore assumed not to be scarlatinal strains. Nine filtrates were incapable of eliciting a positive skin test in dilutions of 1:10 or over.

TABLE 1
TESTS OF 16 FILTRATES OF CULTURES OF HEMOLYTIC STREPTOCOCCI FOUND TO BE SCARLET
FEVER STREPTOCOCCI

Tests	Number Subjects	Size (Mm.) and Intensity of Skin Reaction to—		
		Standard Scarlet Fever Toxin	Filtrates	Filtrates Plus Antitoxin *
1			Filtrate WS 1:1000.....plus antitoxin 1:10	
	1	18 × 15, moderate	10 × 8, moderate	0
	2	14 × 13, moderate	9 × 9, moderate	0
	3	20 × 12, moderate	12 × 7, moderate	0
2			Filtrate MB 1:500.....plus antitoxin 1:10	
	1	20 × 16, moderate	13 × 9, moderate	0
	2	24 × 22, bright	25 × 18, bright	0
3			Filtrate AC 1:150.....plus antitoxin 1:50	
	1	10 × 8, faint	18 × 10, faint	0
	2	25 × 21, moderate	22 × 18, moderate	8 × 6, faint
4			Filtrate CS 1:100.....plus antitoxin 1:50	
	1	15 × 10, moderate	33 × 25, bright	6 × 6, faint
	2	20 × 13, moderate	18 × 16, moderate	0
5			Filtrate JK 1:100.....plus antitoxin 1:50	
	1	20 × 15, moderate	13 × 13, moderate	8 × 7, moderate
	2	11 × 10, faint	8 × 6, faint	0
6			Filtrate JT 1:100.....plus antitoxin 1:100	
	1	22 × 12, moderate	12 × 12, moderate	0
	2	18 × 18, moderate	15 × 13, moderate	0
7			Filtrate IM 1:50.....plus antitoxin 1:50	
	1	20 × 13, moderate	13 × 12, moderate	6 × 5, faint
	2	23 × 18, bright	17 × 12, moderate	0
8			Filtrate EG 1:50.....plus antitoxin 1:100	
	1	24 × 16, moderate	18 × 15, moderate	0
	2	30 × 22, moderate	21 × 20, bright	0
9			Filtrate BB 1:50.....plus antitoxin 1:100	
	1	18 × 11, moderate	21 × 18, moderate	4 × 4, moderate
	2	21 × 14, moderate	15 × 13, moderate	10 × 6, moderate
	3	14 × 11, moderate	15 × 10, moderate	4 × 4, faint
10			Filtrate MA 1:20.....plus antitoxin 1:50	
	1	20 × 18, bright	8 × 6, moderate	0
	2	22 × 18, bright	25 × 25, bright	0
11			Filtrate FA 1:20.....plus antitoxin 1:50	
	1	15 × 15, moderate	12 × 10, moderate	0
	2	12 × 10, moderate	10 × 8, faint	0
	3	20 × 18, moderate	15 × 12, moderate	10 × 8, very faint
12			Filtrate JR 1:20.....plus antitoxin 1:50	
	1	15 × 13, moderate	23 × 18, moderate	7 × 6, moderate
	2	22 × 16, moderate	25 × 16, moderate	0
	3	18 × 16, moderate	12 × 9, moderate	6 × 5, moderate
13			Filtrate MK 1:10.....plus antitoxin 1:50	
	1	20 × 14, moderate	15 × 14, moderate	0
	2	15 × 12, moderate	12 × 11, moderate	0
14			Filtrate MG 1:10.....plus antitoxin 1:50	
	1	16 × 14, moderate	35 × 28, bright	18 × 10, moderate
	2	10 × 10, faint	35 × 35, bright	10 × 8, faint
15			Filtrate GW 1:10.....plus antitoxin 1:50	
	1	23 × 17, moderate	20 × 15, moderate	0
	2	22 × 17, moderate	20 × 16, moderate	0
16			Filtrate LR 1:10.....plus antitoxin 1:50	
	1	23 × 20, bright	24 × 23, moderate	0
	2	18 × 14, moderate	11 × 10, moderate	5 × 4, moderate

0 = no reaction.

* Control tests with antitoxin alone in all instances produced no reactions.

Results of Dick Tests.—In 24 of the patients with hemolytic streptococci in the throat the Dick test was made, the others having left the hospital before the reactions were read. Seven (24.1%) were susceptible to scarlet fever. In the group without hemolytic streptococci in the throat 33 of 68 (48.5%) reacted positively to the Dick test. It is noteworthy that in six of the seven with hemolytic streptococci in the throat who were also found susceptible to scarlet fever, the streptococci showed no toxin production in a 1:10 dilution. The other patient harbored a scarlet fever streptococcus of very weak toxin-producing power, one giving a reaction first in a 1:10 dilution.

TABLE 2

TESTS OF FOUR FILTRATES OF CULTURES OF HEMOLYTIC STREPTOCOCCI CAUSING A POSITIVE SKIN REACTION NOT NEUTRALIZED BY SCARLET FEVER ANTITOXIN

Tests Number Subjects	Size (Mm.) and Intensity of Skin Reaction to—		
	Standard Scarlet Fever Toxin	Filtrates	Filtrates Plus Antitoxin *
1		Filtrate EB 1:100.....plus antitoxin 1:25	
1	18 × 15, moderate	0	0
2	15 × 14, moderate	12 × 10, moderate	15 × 15, moderate
3	15 × 13, moderate	16 × 15, moderate	17 × 17, moderate
4	23 × 18, moderate	0	0
2		Filtrate SS 1:50.....plus antitoxin 1:50	
1	20 × 14, moderate	0	0
2	18 × 16, moderate	10 × 10, moderate	12 × 10, moderate
3	30 × 25, moderate	18 × 15, faint	18 × 12, faint
4	15 × 13, moderate	18 × 17, moderate	15 × 14, moderate
3		Filtrate J L 1:50.....plus antitoxin 1:50	
1	25 × 16, moderate	13 × 12, moderate	12 × 10, moderate
2	23 × 15, bright	13 × 13, bright	15 × 15, bright
3	15 × 13, faint	10 × 10, faint	13 × 8, faint
4		Filtrate MF 1:50.....plus antitoxin 1:50	
1	21 × 18, bright	7 × 6, moderate	7 × 6, moderate
2	21 × 17, faint	8 × 6, faint	8 × 6, faint
3	15 × 14, bright	0	0

0 = no reaction.

* Control tests with antitoxin alone gave no reaction.

Simultaneous Occurrence of Diphtheria and Scarlet Fever.—That the simultaneous occurrence of diphtheria and scarlet fever is not uncommon is shown by the fact that the combination was encountered three times in the present series. In one patient admitted with a diagnosis of diphtheria a rash that could be blanched by scarlet fever antitoxin developed. He later desquamated. One patient had been admitted with the diagnosis of scarlet fever. He subsequently developed diphtheria, supposedly due to cross infection. One patient had entered with a diagnosis of scarlet fever. Eleven days later he had a rash diagnosed measles, and three days later diphtheria was diagnosed. All three patients had scarlet fever streptococci in their throats when cultured.

Conclusions.—Infection with scarlet fever streptococci of comparatively low toxin production may account for the development of a negative Dick test during the course of acute infections diagnosed clinically or culturally as diphtheria.

It is possible that some of the cases reported were scarlet fever occurring in diphtheria carriers and diagnosed diphtheria because of positive cultures.

The results reported indicate the advisability of individual isolation to prevent cross infection with hemolytic streptococci in diphtheria wards.

SUMMARY

Hemolytic streptococci were cultured from the throats or noses of 29 of a series of 100 patients who were admitted with the diagnosis of diphtheria to hospitals for contagious disease in Chicago.

Sixteen, or 55.2%, of these strains of hemolytic streptococci, were identified as scarlet fever streptococci by the toxin neutralization method.

The strength of the scarlatinal toxin produced by these strains varied from 100 to 10,000 skin test doses per cc. It was less than 2,000 skin test doses per cc. in 14 of the 16 strains.

Thirteen, or 44.8%, of the strains of hemolytic streptococci isolated did not produce any demonstrable scarlet fever toxin.

PREPARATION OF SALMONELLA PULLORUM ANTIGENS FOR COMPLEMENT FIXATION TESTS

L. D. BUSHNELL AND C. B. HUDSON

*From the Department of Bacteriology of the Kansas Agricultural Experiment Station,
Manhattan*

During the past year the authors, in attempting to solve the problem of the carrier state in *Salmonella pullorum* infection in the domestic fowl by a comparison of the agglutination and complement fixation tests, discovered that one of the difficulties in technic was the lack of a proper antigen for complement fixation. The antigens usually recommended did not appear to act satisfactorily in this test and several variations in method of preparation were tried as reported below. Bacterial antigens for the complement fixation test comprise the following: whole bacteria and their products in the culture in which they have been grown; suspensions of the bacterial cells in an undisrupted condition but freed of the products of growth; suspensions of the disrupted bacterial cells; and filtrates and washings of the cells. The most difficult part of the technic of making a complement fixation test is the preparation of a stable antigen, one which will retain its antigenic property for a long period of time and still not become anticomplementary. Such an antigen is here described.

Cultures.—The cultures used in this study were typical of those originally described by Rettger and Stoneburn¹ and numerous other workers. From a collection of 83 cultures isolated from different sources and described by Bushnell, Hinshaw and Payne,² three were selected for special study. These were not selected because of any special characteristic but because they made suitable antigens for the agglutination test. Two of these were of the A type and one of the B type as described by Hadley.³ We have since discarded the B type.

Numerous methods of preparation of antigens were tried but the results on the use of cultures and suspensions of untreated organisms gave results which were not satisfactory. The antigens either were not highly antigenic, or were anticomplementary, or hemolytic. In order to overcome these difficulties the suspensions were treated as subsequently described.

Culture Medium.—The medium finally selected for the growth of the cultures was composed of the following ingredients.

Peptone (Parke, Davis).....	15 Gm.	Agar	18 Gm.
Beef extract	3 Gm.	Sodium citrate	2 Gm.
Sodium chloride	5 Gm.	Water (distilled)	1,000 cc.

Received for publication, July 29, 1927.

¹ Storrs Agric. Exper. Sta. Bull. 60, 1909.

² Kansas Agric. Exper. Sta. Tech. Bull. 21, 1926.

³ Rhode Island Agric. Exper. Sta. Bull. 172, 1917.

The mixture was boiled over a free flame for 10 minutes until the agar had dissolved and the loss due to evaporation restored. The medium was allowed to cool somewhat and adjusted to P_H 7.2 using bromthymol blue as an indicator. The medium was then placed in Kolle flasks without filtration, and sterilized for 20 minutes at 15 pounds pressure. This medium produced a constant and luxuriant growth of this organism. The P_H of 7.2 gives the most luxuriant growth on this medium. For long continued growth a lower P_H is best since the organisms render the medium slightly alkaline after a few days.

The addition of the sodium citrate improves the growth of the organism by preventing the precipitation of the phosphates during sterilization. If the phosphates are precipitated and filtered out the growth is considerably reduced.

Time of Incubation.—The cultures were grown at 37 C. for 24 hours, young cultures are superior to older cultures for antigens and the maximum growth has taken place at this time.

Preparation of the Antigen.—The growth was washed off from the medium in each flask with 10 cc. of sterile physiologic salt solution made of double distilled water and chemically pure sodium chloride. The suspensions from the several flasks were pooled and shaken vigorously for several minutes to

TABLE 1
THE VARIABILITY OF ANTIGENIC ACTION AND THE EFFECT OF WASHING WITH ETHER IN
COMPLEMENT FIXATION TESTS WITH IMMUNE BIRD SERUM *

Antigen	Degree of Fixation: Complete (4) to Absent (0)						
	Cc. of Antigen						
	0.02	0.05	0.10	0.20	0.30	0.50	0.70
Not properly washed							
1.....	0	0	1	2	3	4	4
2.....	0	0	0	0	1	3	4
3.....	0	0	1	2	4	4	4
Properly washed							
4.....	2	4	4	4	4	4	4
5 and 6.....	4	4	4	4	4	4	4

* Controls: nonimmune serum with each antigen gave only negative (0) results.

give a uniform mixture. The antigen was then filtered through tightly packed glass wool to remove extraneous materials and clumps of bacteria and transferred to 50 cc. centrifuge tubes and centrifugated until the supernatant fluid was nearly clear (45 minutes to 1 hour at high speed). The supernatant fluid was discarded as it was always found to be too anticomplementary for use. To the sediment (about 1 cc. volume) was added 10 cc. of ether. The sediment was thoroughly stirred at frequent intervals for four hours. The ether extract was discarded and 10 cc. fresh ether added and allowed to act with frequent stirrings for 2 hours. The ether was again discarded and the tubes placed in the incubator until the odor of ether had disappeared. To each tube were added 10 to 15 cc. of salt solution and the organisms brought into uniform suspension by drawing into a pipet and blowing out rapidly 40 to 50 times. More salt solution is added and mixing continued until the turbidity of the bacterial cell suspension was the same as a McFarland nephelometer tube 3. These antigens are not anticomplementary in 1.5 cc.

Table 1 shows the titrations of antigens thus prepared by suspending in salt solution after washing in ether. The results were obtained by using serum of birds which had been immunized to the organism under

test, or by use of serum of naturally infected birds. The examples given are a few taken from a large series.

It may be noticed that the washing considerably increased the antigenic action and greatly reduced the anticomplementary action of these antigens. The first washings in salt solution are always highly anticomplementary while the ether extract is often found to be somewhat so. The emulsification of the bacteria in the salt solution is of great importance. If they are not made completely homogeneous the antigenic action is considerably reduced. More than a uniform suspension seems to be necessary for good results. It may be that something is washed from the cell by this process as will be described later under the heading "ectoantigens."

Extraction with alcohol, acetone and benzene appear to give about the same results as those obtained with ether. By use of acetone the water is rapidly removed from the cells and the residues dry very rapidly. It has one disadvantage that it leaves the cell clumped in hard masses which are difficult to bring into a uniform suspension.

Ectoantigens.—The term "ectoantigens" was used by Ferry and Fisher ⁴ to designate an antigenic substance which is outside the bacterial cell. Such an antigen should have several advantages in the complement fixation test. In many bacterial antigens the antigenic dose is so low that large amounts of heavy suspensions are required. These large doses cause such heavy clouding as to mask the hemolysis in some cases, and render accurate readings difficult. The so-called "ectoantigens" are water clear, and while they could not be used for the agglutination test, they have many advantages for complement fixation.

Preparation: The mass of cells obtained by centrifugation was washed once with salt solution, centrifugated again, and the supernatant fluid discarded. The sediment of cells amounts to about 1 cc. volume. To this sediment are added 7 cc. of salt solution and the cells emulsified thoroughly with a pipet, then 13 cc. of salt solution are added and the washing process continued by sucking into a pipet and blowing out for 50 times. The suspension is again centrifugated at high speed and the supernatant fluid, which is practically cell-free and water clear, is used as the antigen.

This process gives an antigen which is highly antigenic and not anticomplementary in 1.5 cc. amounts. It is especially valuable because it is very clear and does not mask the reading of the tests. The sediment remains anticomplementary after such treatment. Table 3 illustrates the action of several antigens made in this way.

⁴ J. Lab. & Clin. Med., 1925, 10, p. 817.

For all the antigens made by this method from typical strains of the organisms, 0.03 cc. constitute the antigenic dose when tested with a positive serum. The antigens are not anticomplementary in 1.5 cc. amounts and complement is not fixed in the presence of negative serum. The bacterial cell is not essential in antigens made from *S. pullorum*. It is necessary, however, to wash the cells very carefully in order to remove the antigenic substance and bring it into solution.

The following experiment shows that a fairly large amount of antigen may be prepared from a comparatively small amount of cell

TABLE 2
ANTIGENIC ACTION OF CELL-FREE EXTRACTS OF *SALMONELLA PULLORUM* IN COMPLEMENT FIXATION TESTS WITH IMMUNE BIRD SERUMS *

Antigens	Degree of Fixation: Complete (4) to Absent (0)	
	Cc. of Antigen	
	0.02	0.05 to 0.50
1, 2 and 3.....	4	4
4.....	3	4
5.....	2	4

TABLE 3
DISSOCIATION OF ANTIGENIC SUBSTANCE FROM *SALMONELLA PULLORUM* CELLS BY WASHING IN PHYSIOLOGIC SALT SOLUTION: COMPLEMENT FIXATION TESTS WITH IMMUNE BIRD SERUM *

Washing Number	Degree of Fixation: Complete (4) to Absent (0)		
	Cc. of Washing Used as Antigen		
	0.02	0.04	0.10 to 0.50
1.....	3	4	4
3.....	3	4	4
5.....	2	4	4
7.....	1	3	4
10.....	1	3	4

* Controls: nonimmune serum with each antigen gave only negative (0) results.

substance. A mass of about 1 cc. of cells was washed as above described and centrifugated and washed 10 consecutive times and each washing tested separately for its antigenic action. Table 3 shows the tests as obtained by use of these washings. By this process of washing the cells repeatedly the antigenic substance is gradually reduced in amount. The first washing showed the antigenic dose to be 0.04 cc. while the tenth washing showed an antigenic dose of 0.10 cc. This table also indicates that the antigenic substance is removed from the cell with some difficulty and that it will dissociate only to a slight extent before an equilibrium is reached. This point is still under study in this laboratory.

In order to test the stability of this product under various conditions of treatment 120 cc. of the ectoantigen were prepared and divided into 4 equal lots. The antigenic titer of each was determined and all stored at about 6 C. and tested again after 2 and 8 months. The results of these tests are shown in table 4 which shows that the antigenic substance of *S. pullorum* is not altered in its ability to react with the specific anti-serum when subjected to the treatments mentioned. The long storage did not increase the anticomplementary action of the antigen as is so commonly the case of cell suspension.

TABLE 4
EFFECT OF VARIOUS TREATMENTS OF "ECTOANTIGEN" ON COMPLEMENT FIXATION TESTS
WITH IMMUNE BIRD SERUM *

Antigen Lot	Date of Test	Degree of Fixation: Complete (4) to Absent (0)	
		Cc. of Antigen Used	
		0.02	0.05 to 0.50
A	6/5/26	2	4
B		2	4
C		2	4
D		2	4
A	8/3/26	1	4
B		1	4
C		2	4
D		2	4
A	2/5/27	2	4
B		1	4
C		2	4
D		2	4

* Control tests with normal serums were negative (0)

A, not heated; preserved with 0.5% phenol. B, heated 100 C. for 30 minutes on 3 successive days. C, autoclaved for 30 minutes at 20 pounds pressure. D, filtered (Berkefeld), and filtrate kept aseptically.

SUMMARY

The described method of preparing antigens has given a satisfactory product to be used for the complement fixation test. The cell suspension washed carefully with ether is satisfactory in its antigenic action and is not anticomplementary, but causes a turbidity which makes tests difficult to read. The salt solution washings of the bacterial cells are antigenic and not anticomplementary after the second treatment. A very vigorous washing is necessary to cause strong dissociation of this product.

An unfiltered medium containing sodium citrate gives a more luxuriant growth than a medium which has been filtered. The citrate salt holds the phosphates in solution during sterilization.

COMPLEMENT FIXATION AND AGGLUTINATION TESTS FOR SALMONELLA PULLORUM INFECTION

L. D. BUSHNELL AND C. B. HUDSON

*From the Department of Bacteriology of the Kansas Agricultural Experiment Station,
Manhattan*

For several years we have been testing domestic fowl for bacillary white diarrhea without being able to decide on the exact standard to indicate actually dangerous infection of the carriers. There are many low reacting birds which have recovered from the disease and are immune. The serum from others may give nonspecific reactions because of certain intercurrent infections not of a dangerous character, while still others whose serum reacts with the *Salmonella pullorum* antigen only in low dilutions are known to be actual carriers of the bacillary white diarrhea organisms and to eliminate them through the egg. Some of these eggs hatch and cause heavy losses of chicks from normal hens. In an attempt to determine which birds are carriers and of danger to the remainder of the flock extensive experiments were conducted during the past year in comparing, among others, the agglutination and the complement fixation tests of serums from a flock at this station. We have found few comparative tests of this type and in those mentioned in the literature it has been difficult to make a direct comparison of the two tests on the same dilution of the serum.

Wall¹ examined the serum of 1100 animals for infectious abortion with both tests and found a close agreement between the two. He found the agglutination test alone to be a satisfactory method of diagnosis but considered it subject to larger error than the complement fixation test. Of 278 tests made on 75 cows, 247 were positive to both tests, 12 negative to both tests, 16 positive to the agglutination test alone and 3 positive to the complement fixation test alone. Thus of a total 263 serums positive by the agglutination test, 94.2% were confirmed by the complement fixation test. The author examined 106 cows in which no abortion had occurred and assumed that any reactions on this group were errors. With the agglutination test 5.7% were reactive, and with the complement fixation test 0.9% were reactive. The percentage of error was calculated by the formula $\frac{5.7\% \times 0.9\%}{100} = 0.05\%$, which is a close correlation.

100

Surface² states that there is a close agreement in the two tests for infectious abortion in cattle. In testing 181 animals for this disease by both methods he found that 17 showed a positive agglutination but no fixation of complement

Received for publication, July 29, 1927.

¹ Ztschr. f. Infektionskr. d. Haustiere, 1911, 10, pp. 23, 133.

² Kentucky Agric. Exper. Sta. Bull. 166, 1912.

in serum dilutions of 1:20. Only six gave agglutination but no complement fixation in dilutions of 1 to 50. On the other hand there was only one case in which the serum showed complement fixation but no agglutination. He considered that the complement fixation test is the more reliable of the two.

Garbat³ in making a comparison of the complement fixation and agglutination test in typhoid fever frequently found the complement fixation test as frequently positive as the agglutination test and of great corroborative value. Of 37 cases reported, 24 were positive to the agglutination, and 23 to the complement fixation test.

Huddleson⁴ compared the two tests for abortion in cattle. Of 258 tests, 55 were strongly positive, 2 weakly positive, and 181 negative in the complement fixation test with 0.1 cc. of serum. With the agglutination test, 15 were strongly positive, 7 weakly positive and, 208 negative with 0.1 cc. of serum. In only two cases was a reaction obtained which was not confirmed by the complement fixation test. Of the 181 negative in the complement fixation test all but 2 were confirmed by the agglutination test. There were, however, 45 reactions to the complement fixation test which were negative to the agglutination test. These results indicate that the complement fixation test is more dependable and delicate than the agglutination test.

Povitzky⁵ compared the tests on 1890 cases for the diagnosis of glanders. With the complement fixation test there were 254 positive, 21 suspected, 300 doubtful and 1315 negative. With the agglutination test there were 262 positive, 171 suspected, 291 doubtful and 1166 negative. These results show that the agglutination test gave positive and suspected reactions in a larger proportion than the complement fixation test. The doubtful reactions occurred in about the same number in the two tests.

Huddleson⁶ reported on a study of 109 blood samples studied by both tests. There was general agreement in the two tests, 85 were positive to both tests, 15 positive by the complement fixation test alone and 9 negative to both tests. None were positive to the agglutination test alone. Since these results were obtained on inoculated animals a greater specificity of the complement fixation test is indicated.

Buck and Creech⁷ reported a total of 960 bi-monthly tests on 34 heifers. Of these 391 gave positive reaction to both tests, 353 were positive to the agglutination test alone and 288 were negative to both tests. In no case was there a positive reaction with the complement fixation test and negative with the agglutination test. Those reactions obtained with the agglutination test which were not confirmed by the complement fixation test were mostly slight or partial reactions and varied greatly from time to time. These reactions are therefore questionable. They concluded that these two tests are of about the same value. The complement fixation test is more decisive, leaving a smaller number of suspected cases.

Boerner and Stubbs⁸ reported on a comparison of the two tests on serum from 3948 cattle. They found that the agglutination test showed a higher percentage of borderline cases but in over 95% of the cows the reactions obtained by one test were confirmed by the other. They considered that the reactions obtained with the complement fixation test are more distinctly positive or negative than those obtained with the agglutination test.

³ Am. J. M. Sc., 1914, 148, p. 84.

⁴ Michigan Agric. Exper. Sta. Tech. Bull. 32, 1916.

⁵ J. Immunol., 1918, 3, p. 463.

⁶ Michigan Agric. Exper. Sta. Tech. Bull. 55, 1922.

⁷ J. Agric. Research, 1924, 28, p. 607.

⁸ J. Am. Vet. M. A., 1924, 18, p. 425.

EXPERIMENTS WITH BIRD SERUMS

The usual technic of the complement fixation and macroscopic agglutination tests were followed throughout and all precautions taken to control errors.

Antigens.—The antigens were usually prepared from three typical cultures of *S. pullorum* as described by Bushnell, Hinshaw, and Payne.⁹ They were grown for 24 hours on meat extract agar containing 2 grams of sodium citrate per liter to prevent precipitation of phosphates during sterilization. This medium was used at a P_H of 7.2 and unfiltered in Kolle flasks. In some of our work we have used a synthetic medium described by Melick¹⁰ for the preparation of antigens. This medium does not contain any organic nitrogen except asparagine and ammonium lactate. This medium gives a moderate growth of the organisms on the bottom of the tube. The antigens prepared from this medium are of interest because they are not anticomplementary. Also growth on chicken-meat infusion agar is not highly anticomplementary after careful washing with salt solution.

The organisms were suspended in salt solution, centrifugated, extracted twice with ether, and resuspended in salt solution after removing the ether. The cells were thoroughly suspended and standardized to a turbidity of McFarland nephelometer tube 3.

The same antigen was used for both the agglutination and complement fixation test. A convenient amount of 0.03 cc. was chosen as the antigenic dose. This was approximately 10 antigenic units and not more than one-fifth of the anticomplementary dose. For the agglutination test this amount of antigen was diluted to 2 cc. and about corresponded in density to tube 1 McFarland nephelometer.

No preservative was added to the antigen before making the complement fixation test but after diluting, 0.3% phenol was added to the antigen used for the agglutination test since these were incubated for 24 hours before readings were made.

In all, 230 tests were made on 98 birds, some of which were known to be carriers and others were considered negative by all tests. The birds were considered to be negative because of negative agglutination and pullorin tests because chicks hatched from their eggs had not developed the disease. The birds which were considered to be infected, had reacted positively several times to the agglutination and the pullorin tests, and the organism had

⁹ Kansas Agric. Exper. Station Tech. Bull. 21, 1926, p. 85.

¹⁰ J. M. Research, 1923, 42, p. 405.

been isolated from several of their eggs, dead embryos and chicks which died of the disease. A few only had highly reacting serums: most serums reacted persistently in dilutions of 1:40 to 1:160. A long series of agglutination tests, extending in some cases over a period of two years, had been made on most of these birds. By numerous tests 41 of the birds studied were considered to react and 57 not to react. Most of the tests reported in this paper were made on these birds, at intervals of one to several months. In this we may consider each test as a separate comparison. There are then 106 tests from reactive birds, and 124 from unreactive. Of the 106 tests made on serum from birds which were considered to be reactive, 95 (89.6%) were positive to both the agglutination and complement fixation tests in serum dilutions of 1:50. Of the tests made on the serum of negative birds, 113 (91.1%) were negative to both tests. There were 11 (10.4%) of the tests from positively reacting birds which were missed in both the tests, 9 by the agglutination and 11 by the complement fixation test. Twelve, or 9.7%, of the tests on the serum of unreactive birds were positive by one or the other of the tests, seven by the agglutination test and nine by the complement fixation test, and seven by both tests.

From the above data we may conclude that the agglutination test is slightly more accurate than the complement fixation test but the differences are insignificant. There were 10.4% of actual carriers missed and 9.7% of the noncarriers reacted to both of the tests when a 1:50 dilution of the serum was used. Of the 97 reacting positively to the agglutination test, 97.9% were confirmed by the complement fixation test. Of the 113 negative reactions to the agglutination test 96.5% were confirmed by the complement fixation test.

If lower dilutions of the serum could have been used there would have been fewer reactive birds missed, and more that were not reactive considered as reactive. Both tests are in error to nearly the same extent and in the same direction. The removal of noncarriers from a flock is of little significance but having even 1% of a flock reactive may be fatal to a season's hatch.

Each test presents certain difficulties. In the agglutination test there is the time element for incubation, the fat-like material present in certain serums, the growth of phenol-fast organisms which may develop in the agglutination tubes, and the difficulty of interpreting the reaction in borderline cases. The complement fixation test also presents certain difficulties. The most important is the strong anticomplementary action of the fowl serum. This renders the use of dilutions of less than 1:50

impossible in many cases. It is also impossible to remove this by inactivation with heat because the complement fixing substances are removed at the same time. The serum is rarely hemolytic enough to cause trouble¹¹ since the anticomplementary action of the serum of the fowl makes the use of low dilutions impossible. We were unable to obtain comparisons in dilutions of 1:20 which we believe gives the most accurate results with the agglutination test.⁹ In a few serums there appears to be very high precipitin content which interferes with the fixation. This does not seem to be entirely a prozone reaction since it does not disappear in proportion to the dilution, but the rapid sedimentation of the antigen inhibits fixation of complement.

In 12 of these cases, 10 of which were classified as weakly positive to the agglutination test and two of which were classified as negative to the complement fixation test and strongly positive to the agglutination test, very rapid agglutination or precipitation took place. The titer limits for these serums were found to be between 1,250 and 2,500. In the complement fixation test a fine granular precipitate developed within a few minutes. This precipitation of the antigen prevented complete fixation of the complement even when shaken at frequent intervals. In all cases the hemolytic control tubes on the serum showed no reaction. A similar precipitate occurred with the water clear antigen described in a previous article.^{11a}

DISCUSSION

The results of this study indicate that the anticomplementary substance of *S. pullorum*, which cannot be removed by washing with physiologic salt solution, may be due to the medium on which it is grown. When the organism is cultivated on beef-extract agar it is anticomplementary and remains so even after ten washings with salt solution. The washings of these cells, however, contain considerable amounts of the antigenic substance but are not anticomplementary after the second treatment. When grown on a synthetic medium, or on chicken-meat infusion agar, antigens are obtained which can be washed free of the anticomplementary substances by means of salt solution. Organisms grown on beef extract medium may be freed of their anticomplementary substances by washing the cells with ether or some other fat solvent.

The medium on which the organisms are grown does not seem to affect the antigenic action of antigens prepared by the same technic.

¹¹ J. Agric. Research, 1924, 27, p. 709.

^{11a} Bushnell and Hudson: J. Infect. Dis., 1927, 41, p. 383.

In the preparation of antigen of high antigenic titer and low anti-complementary action complicated methods are unnecessary. Antigens may be prepared by simply extracting the cells with ether, acetone or benzene and resuspending in salt solution as described.

The highly antigenic substance which is extracted by salt solution and termed "ectoantigen" after Ferry and Fisher¹² is of unknown chemical nature. It appears to dissociate from the cell with considerable difficulty and reaches an equilibrium in the solution in rather low concentrations. This is indicated by the fact that it continues to leave the cells and collect in the washings to about the same amount even after several treatments. These substances are not anticomplementary in amounts of 1.5 cc.

The antigenic compounds are thermostable, resisting temperatures of 100 C. for 30 minutes on three successive days and autoclaving for 20 minutes at 30 pounds pressure. It can, therefore, be preserved in sterile form and does not change in its antigenic or anticomplementary action for at least eight months.

The complement fixation tests are conducted to determine if possible what dilution of serum to consider as indicating a carrier condition in the domestic fowl. From this work the test cannot be considered as of more value than the agglutination test although it is of value in confirming it. In serums with extremely high titers a precipitate interferes with the complement fixation test and the serum of the fowl is so anticomplementary that its use in dilutions below 1:50 is prohibited, thus rendering it of little value in the diagnosis in cases of low reactions. The test may be used to advantage in the so-called fatty serums with which it is difficult to obtain satisfactory agglutination tests.

Of the 89 birds on which two or more tests were run, only four were positive after an initial negative, and seven negative after an initial positive. In all these cases the initial reaction was weakly positive. With birds giving an initial complete reaction with 0.04 cc. of serum the results were variable, although, for the most part the negative birds remained negative and the positive birds positive. We believe that this is due to the uniformity of the antigen which we used in testing and that much of the variation in tests observed by other investigators can be eliminated by more care in the preparation of antigen and interpretation of the tests.

¹² Brit. J. Exper. Path., 1924, 5, p. 185.

One of the greatest difficulties in using the complement fixation test for the diagnosis of carriers of bacillary white diarrhea is the marked anticomplementary action of the serums of the domestic fowl. The serum cannot be inactivated by heating at 50 C without destroying most of the complement fixing bodies which may be present.

In 35% of 400 tests the serum showed anticomplementary action in 0.05 cc. amounts. If some methods could be devised to overcome this factor the results obtained with complement fixation would be more dependable.

CONCLUSIONS

A highly antigenic substance which may be separated from the cells of *Salmonella pullorum* by washing with salt solution. This substance is soluble in salt solution and is thermostable, and does not act freely in complement-fixing tests unless it is separated from the cells. All cultures of the organisms are not equally rich in this substance.

The complement fixation and agglutination tests have about the same value in testing for carriers of *S. pullorum*. A combination of the two tests is more decisive, leaving a smaller number of questionable cases. The complement-fixing test cannot be used with low dilutions of serum and is of no special value in detecting actual carriers of the organisms.

THE INCIDENCE OF CARRIERS OF *B. AERTRYCKE* (*B. PESTIS CAVIAE*) AND *B. ENTERITIDIS* IN WILD RATS OF SAN FRANCISCO

K. F. MEYER AND K. MATSUMURA

*From the George Williams Hooper Foundation for Medical Research, University of California
Medical School, San Francisco*

Through the observations of Salthe and Krumwiede,¹ attention has again been called to the possibility of rats or mice acting as the vehicle of transmission of certain types of bacteria responsible for food infection cases. In an outbreak involving 59 cases these workers traced the contamination of the causative food (cream-cornstarch filling of eclairs and crumb cake) to rodent droppings which contained a distinct paratyphoid B type (*B. pestis caviae*). The significance of this conclusive study cannot be underestimated, since for the first time adequate proof has been offered for the many theoretical considerations which associated rodents with the contamination of foodstuffs. Savage and White² in a study of 100 outbreaks of food poisoning state that rodents acting as vehicles of specific bacteria could never be excluded, but in only one instance was the evidence of a positive character. In this outbreak from one out of three mice caught on the premises adjoining those where the infected food was prepared a bacillus of the paratyphoid (*Salmonella*) group was isolated. In another group of cases reported by the same investigators the history suggests that the fowl responsible for the cases was infected after dressing for the table and that rats and mice may have been the transmitting agent.

In the outbreak investigated by Salthe and Krumwiede the conditions conducive to a detailed epidemiological examination of the food infection cases were used to full advantage. In the majority of the communities no adequate investigation of food poisoning cases is made or the necessary steps towards the study of the factors responsible are taken only when the supposed peccant food has already disappeared. It is therefore not easy, in fact, for obvious reasons impossible to ascertain in any outbreak of food infection if the contamination came from rodents. However, it is surprising that not more outbreaks have been definitely traced to this source. Uhlenhuth and Hübener,³ Jordan⁴ and Savage⁵ in their summaries on food infections and more recently Willfühl and Wendtlandt⁶ and Spray⁷ in separate papers cite outbreaks which were due to foodstuffs contaminated with rat viruses such as "Ratin," etc. Since it has been established that rats and possibly other animals can, after apparent recovery, continue to carry the bacteria of the viruses and to excrete them for long periods, it is not unlikely that they may and often do gain access to foods used for man.

Received for publication, Aug. 9, 1927.

¹ Am. J. Hyg., 1924, 4, p. 23.

² Medical Research Council Special Report Series No. 92, 1925, p. 47.

³ Handbuch d. pathog. Mikroorganismen, 1913, 3, p. 1005.

⁴ Food poisoning, 1917.

⁵ Food poisoning and food infections, 1920.

⁶ Ztschr. f. Hyg. u. Infektionskrankh., 1921, 94, p. 192.

⁷ J. Am. M. A., 1926, 86, p. 109.

Even if the promiscuous use of such bacterial viruses is restricted by law as attempted in California and in New York State, there still remains the possibility that rats and mice may become infected with certain bacteria of the paratyphoid group "without human intervention" (Jordan). Bainbridge,⁸ probably based on an observation made with Boycott⁹ in which 100 wild rats secured from a country farm succumbed in eight days to an acute *B. enteritidis* infection when brought to the laboratory, expressed the view that the intestinal tract of rats is the "true home" of *B. enteritidis*. Although this statement is probably too sweeping, definite evidence has been produced by Savage and Reed,¹⁰ and Savage and White¹¹ that paratyphoid strains are not natural inhabitants of the rat but that they may be present in many animals. Furthermore, since many of the rats showed positive agglutinins for *B. enteritidis*, these workers concluded that the persistence of the organism was the result of an old infection. The bacilli are associated with a diseased condition; the animals are therefore true "carriers" and not merely intestinal "shedders." Savage and White were, from the material at their disposal, unable to decide whether the infections were derived from bacterial rat virus bait or from natural infection. However, they emphasize that the *B. enteritidis* strains isolated by them from wild rats were highly virulent, and that they differed in no particular from the strains isolated from food poisoning outbreaks.

Spontaneous outbreaks of infectious disease amongst tame rats and mice are not uncommon in the laboratories of this country. In several instances organisms belonging to *B. enteritidis* group have been isolated and serologically identified (Jordan).¹² Concerning the distribution of such infections in wild rats nothing definite is known. Macalister and Brooks¹³ diagnosed as *B. enteritidis* infections by naked-eye signs and by bacteriological tests 19 rats of a series of 15,332 rodents which were examined in connection with the plague control work of Essex County, England. They state that this figure does not represent the degree of prevalence of this disease among the rats since such an examination was not in the scope of the investigation. Meyer and Batchelder¹⁴ encountered in the course of a detailed bacteriologic study of 88 wild rats collected on the Oakland side of the San Francisco Bay region eight rodents which harbored *B. enteritidis* either in the spleen or in the liver. The animals came from a garbage dump on which no dosing with virus had occurred during the past three years. However, since bacterial baits had been used in the region adjacent to the dump it was naturally impossible to state with absolute certainty that the carrier state of the eight rats was due to a natural infection. The finding of *B. enteritidis* carriers among a small group of rodents caught in a restricted area was rather disquieting but the sanitary significance could not be evaluated without extending the survey. For a number of reasons the examination of a larger series of rats appeared desirable. According to the published records only *B. enteritidis* has been found in the tissues of wild rats caught in Great Britain and the United States. In this connection the statement of Savage and White that *B. aertrycke* is the widespread cause of enteritis in mice, guinea-pigs and other rodents, but does not commonly occur in rats is of considerable interest. In fact Jordan (p. 315)¹² clearly states that no established instance of rat infec-

⁸ Lancet, 1912, 1, p. 849.

⁹ J. Hyg., 1911, 11, p. 443.

¹⁰ Ibid., 1913, 13, p. 343.

¹¹ Ibid., 1923, 21, p. 258.

¹² J. Infect. Dis., 1925, 36, p. 309.

¹³ J. Hyg., 1914, 14, p. 322.

¹⁴ J. Infect. Dis., 1926, 39, p. 386.

tion with the aertrycke type has come to his notice. Herz and Trawinski¹⁵ claim the isolation of *B. paratyphosus* A and B from the intestines of wild rats in Austria. The majority of bacteriologically studied food infections in this country and in England are caused by *B. aertrycke* or allied bacteria and the only outbreak definitely linked with rodent droppings was due to an organism of this group.

In the light of these facts one is forced to the following conclusions: either wild rats play a very subordinate rôle in the epidemiology of food infections; or the findings of Savage and White based on a relatively small number of rats in a community in which rat viruses are scattered are probably incomplete. Although a well organized inquiry into the etiologic factors of food poisoning cases in California has thus far failed to supply suitable material for an investigation of the first conclusion. An excellent opportunity to study the diseases of rats is, however, afforded through the continuous and searching rat eradication measures which are in progress on the Pacific Coast. Through the collaboration of Dr. N. E. Wayson in charge of the Plague Control Laboratory, U. S. P. H. S., an estimate of the percentage of paratyphoid-enteritidis infections among wild rats caught in various districts of the City of San Francisco was made during the months of December, 1925, January, May, June and July, 1926. The findings of this survey, which established in wild rats the existence of carriers of *B. enteritidis* as well as *B. aertrycke*, are described in this paper.

Methods.—The rats used in this study were selected from the daily catches which were necropsied at the Plague Laboratory. As a rule the rodents were examined by two groups of experienced workers and only animals in a good state of preservation showing some lesions but not those of plague were taken to the laboratory. An equal number of anatomically normal rats caught in the same district were included in the series.

The selection of the best bacteriologic procedure offered a number of difficulties. The enrichment of the intestinal content in bile, peptone, brilliant green solution according to Havens and Dehler¹⁶ with subsequent plating on eosin-methylene blue agar was unsatisfactory on account of the slight inhibition of the constantly present spreaders. Direct plating of the organs and intestinal content as practised by Miss F. Chess and by Meyer and Batchelder in a previous survey gave frequently negative results. Subsequent enrichments proved the presence of bacteria of the rodent typhoid group. As a result of an extensive study the following economical and reliable procedure was finally adopted: Pieces of the liver and spleen or the whole organs and portions of the intestinal content (colon) were enriched for 12 to 16 hours at 37 C. in a liquid medium of the following composition:

Sugar free veal infusion-peptone broth PH 7.0.....	1000.0 cc.
Brilliant green (Grübler).....	0.5 gm.
1 % alcoholic gentian violet solution (Coleman Bell improved).....	1.0 cc.

¹⁵ Wiener klin. Wchnschr., 1917, 30, p. 524.

¹⁶ J. Lab. and Clin. Med., 1924, 10, p. 238.

The medium was tubed in large test tubes shortly before use. A drop of the enrichment culture was spread over one or several brilliant green, eosin, peptic digest agar plates (Stickel and Meyer). These plates were incubated for 24 hours at 37 C. As a rule the positive plates prepared from the spleen or livers revealed pure cultures of nonlactose-fermenting bacilli. On the plates smeared with the enriched intestinal content various nonlactose-fermenting gram-negative bacilli were encountered. They were roughly classified by slide agglutination tests with specific antisera. Previous studies left no doubt that the digestive tract of rats harbors various types of nonlactose-fermenting rods, which can only be distinguished from the true paratyphoid-enteritidis organisms by agglutination or by extended cultural tests. Suitable colonies of the organisms which were agglutinated by one of the type serums were transferred to agar slants or veal broth, incubated for eight hours and then purified by plating on eosinmethylene blue or plain peptic digest agar. All purified cultures were subjected to biochemical tests in various carbohydrates, lead acetate agar, etc. The final identifications were made by means of macroscopic agglutination tests on formalinized suspensions. Specific rabbit serums prepared according to the method of Andrewes¹⁷ with the type strain, *B. aertrycke* of the Lister Institute and *B.*

TABLE 1
RAT TYPHOID IN SAN FRANCISCO

Organism	Isolated from						Total Number of Infected Rats	Total number of Cultures
	Liver Alone	Spleen Alone	Intes- tine	Liver and Spleen	Liver and Intes- tines	Spleen and Intes- tine		
<i>B. enteritidis</i>	9	10	6	3	2	2	28	32
<i>B. aertrycke</i>	14	7	3	6	1	3	30	34
						Totals.....	58	66
Total number of rats examined.....							775	
Percentage of carriers.....							7.48	

enteritidis of the Reichsgesundheitsamt with titers of 1:20,000 and above were employed throughout the survey. The pathogenicity was tested on kittens and tame rats.

Since the slide agglutination test was used to segregate the various nonlactose-fermenting bacteria it is not unlikely that some of the inagglutinable body strains were not recognized. The large number of cultures which had to be handled rendered a more exhaustive bacteriological examination impractical. In a thorough carrier survey of a small area in the vicinity of the slaughter houses special attention will be paid to some of the details which thus far were not in the scope of the broader investigation.

Results of the Survey.—A detailed account of each individual bacteriological examination is omitted. The significant data are summarized in table 1.

There were, in all, 775 rats examined. From the organs and the intestinal content of 58 rats bacteria of the paratyphoid-enteritidis group

¹⁷ J. Path. & Bact., 1922, 25, p. 505.

were isolated. Contrary to previous findings not only *B. enteritidis*, but also *B. aertrycke* (*B. pestis caviae*) was serologically identified. In fact, the percentage of carriers is slightly higher (3.9%) for the latter than for the former (3.5%). The bacteria were primarily recovered from the liver and spleen; in nine instances both organs of the same animal were infected. In nine rats the specific organisms were found only in the colonic content, while in eight cases they were also demonstrated in the liver or in the spleen. It is evident that over 2% of the rodents examined in this survey were capable of shedding paratyphoid-enteritidis bacilli into the feces. This figure could in all probability have been increased by a systematic study of the small intestines. It is a well known fact that as a rule the typhoid-paratyphoid organism decrease in number from the upper to the lower portion of the intestinal tube. Then again the elective isolation of the enteric bacteria from the colonic content even with the aid of a very selective enrichment fluid is less reliable than the cultivation from the anterior portion of the jejunum or the duodenum. However, since the positive cultures from the liver or spleen, may be regarded as evidences of old infections, it is not unlikely that every positive rat may at any one time excrete the specific bacilli.

It may be mere coincidence that *B. aertrycke* was found more frequently in the liver (21) than in the spleen (10). On the other hand, *B. enteritidis* was isolated in 14 instances from the liver and in 12 cases from the spleen. An attempt was made to correlate the bacteriologic results with the macroscopic postmortem findings. Only 39 of the 58 rats showed lesions in form of stippling of the liver, white, parasitic nodules or small necroses in the liver or in the spleen, while the notes for 19 rats record an absence of definite lesions. In this connection it is important to recall that 50% of the rodents were selected for this study on account of the morbid lesion or lesions which were considered typical for rat typhoid. However, in only one-tenth of these chosen cadavers were the gross changes associated with paratyphoid bacilli. On the other hand a small number of rodents (19) with apparently normal organs harbored these bacteria. For technical reasons it was impossible to examine the blood serum of the "marked" rats for agglutinins. Such tests might have been of some help in the determination of the etiology of the lesions, although Savage and White definitely state that the white spots on the liver were not especially associated with agglutinins for the *Salmonella* strains. Similar deductions regarding the value of

this test were drawn by Ball and Price-Jones¹⁸ who expressed the view that the agglutination test would be useless for identifying the animals infected with *B. enteritidis* in a laboratory epidemic among rats. Since the wild rats trapped in the San Francisco Bay region are subject to various infections¹⁴ it is suspected that the deviations in the internal organs are either in a small percentage of cases healed and sterile lesions of rat typhoid or they are the result of various other unknown causes which should be investigated.

The isolated paratyphoid bacilli fulfilled all the cultural characteristics of the group. They fermented the hexoses, maltose, xylose and arabinose and blackened the lead acetate mediums. Six strains grew as "granular" and two as nongas-producing variants. Thirty-two of the purified strains were promptly agglutinated by a specific *B. enteritidis* serum (titer 1:20,000) to the titer limit. About one half of the cultures gave co-agglutination reactions in the serum dilutions of 1:10 and 1:100. The *B. aertrycke* strains were only clumped by the specific antiserum; co-reactions were recorded in the lower dilutions. A few adsorption tests confirmed the specificity of the reactions.

In the course of this study a number of nonlactose-fermenting gram-negative bacilli which were agglutinated by various antisera and by normal rabbit serum in the dilution of 1:100, were isolated from the intestinal content of many rats. Biochemically these organisms resemble closely the pseudo- or para-gaertner bacilli described by Savage. Without an extended series of cultural or detailed serologic tests these strains may be easily mistaken for true paratyphoid or enteritidis bacilli. The significance of these organisms is by no means clear, and a more detailed study is urgently needed.

The pathogenicity by feeding was tested upon four 8-weeks old kittens and 12 tame rats. For the tests one agar slant of the 24-hour culture was washed off with 5 cc. of broth and the resulting suspension mixed with 240 cc. of fresh pasteurized milk. A portion of this infected milk was placed in the cages of the experimental animals. The drinking water of the rats was contaminated with a suspension of the bacteria prepared from the agar slants.

Every one of the 15 different strains which were tested for pathogenicity by feeding with relatively large doses was virulent. The kittens developed symptoms of profuse watery yellow or blood-tinged diarrhea in from 18 to 24 hours, anorexia, toxemia and loss of weight. As a

¹⁸ J. Path. & Bact., 1926, 29, p. 27.

rule, the symptoms disappeared by the 4th or 5th day and a second feeding of infected milk was then offered. One kitten died on the 2nd day and showed at necropsy a severe enteritis and a general *B. enteritidis* septicemia. One cat tested with *B. aertrycke* was ill for three weeks and was found dead on the 23rd day after the second feeding. No naked-eye lesions were noted and the paratyphoid organisms could not be recovered from the intestines. The remaining two kittens showed no ill effects after the 2nd and 3rd feeding, although the ingested organisms were found in the feces, at necropsy in the 3rd week the gross and bacteriologic findings were negative. Very similar results were obtained on tame rats. Irrespective of the type or origin of the strain severe to moderate symptoms were produced and seven of the twelve rodents succumbed to the infection in from 48 to 72 hours after the 1st or 2nd feeding. The early deaths were probably due to intoxications since the specific organisms were only demonstrated in the empty intestines. On the other hand the two rats which died on the 9th day of the disease exhibited engorged livers and spleens with small and large necrotic areas. Typical *B. enteritidis* were isolated from the internal organ and the intestines. Negative findings were the rule in the animals which recovered and were sacrificed in the 3rd week.

Two strains each of *B. enteritidis* and *B. aertrycke* of rat origin produced soluble heat stabile toxin endowed with the same pharmacologic properties as found in this group of bacteria. These observations leave no doubt that the organisms are highly virulent and that they differ in no particular from the strains isolated from other sources especially food poisoning outbreaks. In this connection one would wish to know more concerning the significance of these microbes in the rodents. Since they were isolated from the internal organs and not only from the intestinal tube it is reasonable to conclude that they are not harmless natural inhabitants, but true tissue parasites. More information concerning this point could have been secured by systematic tests of the serum for antibodies. Even without such evidence it is fairly certain that the rats derived the paratyphoid bacilli from natural infection and not from bacterial baits. *B. aertrycke* is not employed in rodent control work and "Ratin" which contains a bacillus indistinguishable from *B. enteritidis* has not been used in the areas in which the rats have been trapped. It is clear that rat typhoid as a natural disease exists among the rodents of San Francisco and that the 7% of the infected hosts which were bacteriologically recognized represent the carrier-rate in an endemic area. The question naturally arises: is the

carrier incidence widespread and evenly distributed throughout the city? An answer to this question would also decide the important query: does the paratyphoid-enteritidis carrier state endanger the food-stuffs either at the source of production or in the course of distribution? The data which are of some assistance in formulating the answers are presented in table 2.

Reliable information regarding the origin of rats in the city were available for 472 rats. Over 48% of the total came from the vicinity of the slaughter houses (Butchertown). The carrier rate in this group is 6.3%; two thirds of the infections were due to *B. enteritidis*. The groups representing the warehouse, retail and second class residential districts are relatively small, but it is significant that even for these areas a carrier rate of from 4.4 to 8.9% was calculated. A relatively low

TABLE 2
DISTRIBUTION OF RAT CARRIERS OF *B. AERTRYCKE* AND *B. ENTERITIDIS*

Districts	Total Number of Rats	Infected Rats		<i>B. enteritidis</i>	<i>B. aertrycke</i>
		Number	%		
Butchertown	228	18	6.3	13 (72%)	5 (28%)
Wholesale district.....	70	1	1.4	1	0
Warehouse and factories—large retail..	60	3	5.0	2	1
Retail and residential district 2nd class...	67	5	8.9	2	3
Unclassified; scattered small retail and second class residential districts.....	47	2	4.4	0	2
Totals	472	29	6.2	18	11

incidence was established for the wholesale districts. If one excludes from consideration the latter districts it is evident that the carrier rate is nearly the same throughout the city. For numerous reasons it would be of interest to possess comparative data from other cities. The only available information regarding *B. enteritidis* carriers among the rats of a slaughter house was given by Savage and Reed as 8.5%. Unfortunately the data of this survey are of little value for comparison since the two workers admit that it is impossible to exclude rat virus as the original source of infection. Then again a confined area and not large districts of a growing city have been surveyed. From the standpoint of distribution of the rat population San Francisco is probably unique. A vigilant and energetic rat extermination service by traps and poison as a part of the plague control work on the Pacific Coast continuously disturbs and scatters the rodents. Large nesting places with the exception of the refuse dumps are rarely found. Under these conditions the dominating influence of crowding is reduced and the morbidity

and the carrier rate of an epidemic disease like rat typhoid should be small. If the data here presented picture the conditions as they exist in a community with a scattered rat population then it is theoretically reasonable to fear that in other cities or towns with a prolific, undisturbed rodent class a higher morbidity will create a much higher carrier rate. Already in the introduction attention has been called to the connection between rodent typhoid and food infections. It is quite unnecessary to stress the fact that a community with a large number of infected rodents may encourage at the source of production or in the home the accidental contamination of foodstuffs by rat droppings. Further, a modern community with 90% of the buildings perfectly rat proofed is less liable to have the diseases of rodents transmitted directly or indirectly to human beings than a city in which no particular precautions are taken.

If, as seems probable, many of the food infection outbreaks are due to *B. aertrycke* of rodent origin it may be the duty of the public health intelligence service of the future to keep, by continuous sampling of the mice and rats, a close watch of the paratyphoid carrier rate of a certain area or of certain buildings of a community. Such surveys will probably furnish data which may be used to interpret the observations in San Francisco and may help to predict the danger of food infections and food poisoning from rodent sources. Until the many vexed questions associated with such a problem have been settled it is advisable to take every precaution to keep rats and mice from gaining access to foods used for man.

SUMMARY

As a part of a study of certain factors which may or may not be responsible for food infections and food poisoning outbreaks it appeared desirable to establish the existence and the incidence of rodent typhoid in the rat population of San Francisco. A bacteriological examination of 775 rats (*Mus norvegicus*, *decumanus* and *rattus*) revealed 58 rodents which were infected either with *B. enteritidis* (28 cases) or *B. aertrycke* (30 cases). As far as published records permit of deductions, this study records for the first time the occurrence of *B. aertrycke* in wild rats. At least 17, or 2%, of the rodents harbored the specific organisms in the intestinal tube and were capable of shedding highly virulent bacilli into the feces. Only two thirds of the infected rats exhibited lesions in the liver and spleen which might be considered the naked-eye signs of

a preceding infection. Since the animals came from districts in which no rat virus baits had been scattered it is clear that they derived the paratyphoid-enteritidis bacilli from natural infection. The carrier rate is fairly uniform throughout the city and is approximately 6% in the vicinity of the slaughter houses, retail and second class residential districts. It is not unlikely that the conditions found in San Francisco are typical for a city with a vigilant rat extermination service and should not be considered as representative of the prevalence of rat typhoid in other large communities.

THE REACTION OF THE DOG TO THE CONTINUOUS INTRAVENOUS INJECTION OF B. COLI

BACTERIOLOGICAL AND CHEMICAL STUDIES OF THE LYMPH

WILLIAM F. PETERSEN, ERNST F. MÜLLER

AND

WILLIAM BOIKAN

From the Department of Pathology, University of Illinois, College of Medicine, Chicago

During the course of observations on the lymph of normal dogs we have carried out a series of experiments during which a dilute suspension of *B. coli* was continuously injected intravenously over a considerable period of time (6 to 24 hours). In the experiments, we were able to reproduce the clinical picture of the onset and course of an acute, severe, infectious process and by means of the analysis of the lymph, could follow some of the chemical changes coincident with the clinical changes. Inasmuch as the thoracic lymph is derived chiefly from the liver and the splanchnic area, such analysis presumably yields considerable information about the reaction of the splanchnic viscera to the bacterial invasion.

Technic.—The technic of the experiments has been covered fully in previous papers;¹ we may merely mention that the animals were normal dogs, in which a thoracic duct incannulation had first been made under local anaesthesia. A period of recovery was followed by the continuous intravenous injection of *B. coli* (usually 1 slant of a 24-hour culture in 500 cc. Ringer's solution) by means of the Woodyatt pump. The rate of injection is noted with each protocol.

Lymph analysis was made for protein, globulin, sugar, erepsin, potassium and calcium, nonprotein nitrogen and CO₂ combining power. In some experiments urine flow and blood pressure was observed, as well as muscle and rectal temperature, leukocyte count, and respiratory rate. The bacteriology of the lymph was followed in all experiments of this series.

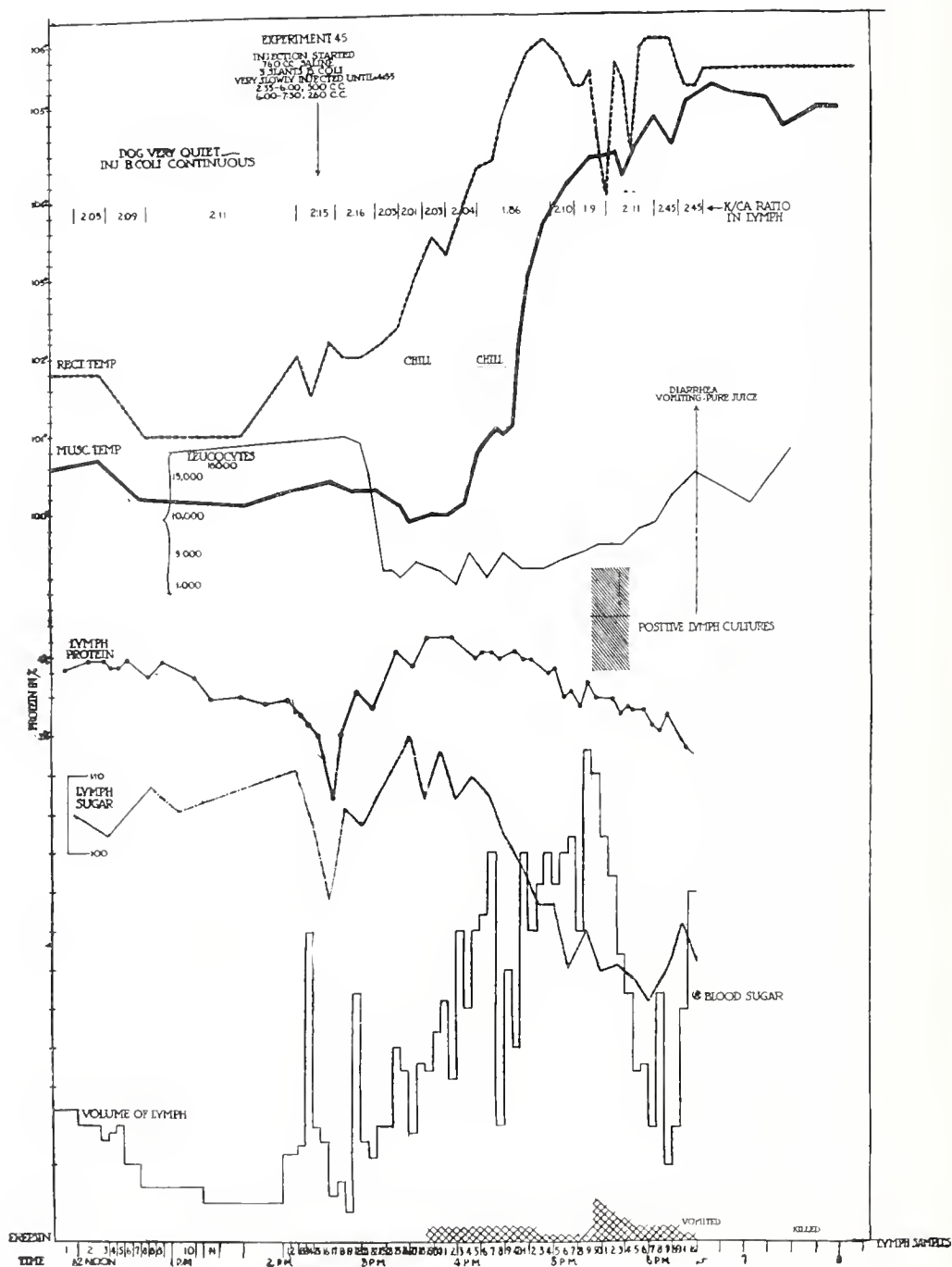
EXPERIMENTS

Chart 1.—A normal quiet animal, weight 9 kilo. During the control period (from noon to 2.35 P. M.) it will be noted that the fluctuations in lymph constituents were within narrow limits. The K/Ca ratio increased gradually (from 2.05 to 2.15) and with it the lymph volume and lymph protein diminished, while the sugar remained practically constant, as did blood leukocytes and temperature.

Received for publication, Oct. 10, 1927.

¹ Petersen, W. F. (and others): *J. Immunol.*, 1923, 8, p. 323. Petersen, W. F., and Plugh, T. P.: *J. Biol. Chem.*, 1925, 63, p. 181; 66, p. 229.

CHART 1



Following the beginning of the injection to about 4.30 P. M. (2 slants of *B. coli* in 500 cc. slowly injected) a gradual diminution of the K/Ca ratio is to be noted (to 1.86) with increase in lymph volume and protein, sugar and erepsin. The leukopenia is marked and it will be noted that the rectal temperature has increased while the muscle temperature lags greatly. We have discussed the significance of this latter observation in other papers.² The muscle temperature declined with the onset of chill.

The rate of injection was then increased (4.55 P. M.). Following a temporary recovery of the K/Ca ratio to 2.10, it declined again to 1.9. At this time apparently maximum damage occurs to the organism. The lymph flow is at its greatest volume, the erepsin concentration becomes greatest, sugar sinks to a low level, a definite break occurs in the rectal temperature (cessation of internal heat production?) and bacteria make their appearance for the first time in the lymph, the cultures remaining positive for a 20 minute period.

A premortal period now sets in with increase in the K/Ca ratio to 2.45. This is due probably to potassium released from injured red blood corpuscles. Peripheral leukocytes increase (peripheral dilatation) and with it a further increase in temperature is checked. The animal was killed at 7:30 P. M.

Chart 2.—A normal dog weighing 8 kilo. During the control period of 45 minutes the only abnormality was apparent in a relatively high leukocyte count (20,000).

The injection (slow) was followed by an immediate drop in the K/Ca ratio (2.04 to 1.69), coincident with an increase in lymph protein and sugar.

This was immediately reversed (to 1.81) and with it sugar diminished as did lymph volume and protein. Leukocytes were held at their original level.

Then for the next two hours (to 2.45) the K/Ca ratio diminishes, lymph volume and protein increases, sugar reaches a maximum level and lymph erepsin is increased. As in the previous experiment, the rectal temperature increases while the muscle temperature lags (note the chill at 2 P. M., and the decline of the muscle temperature with it).

Apart from the transient reversal of the K/Ca ratio to 1.79 at 3 P. M., the ratio declines to a low level of 1.44 and apparently maximum damage is occurring during this time. (The rate of injection was increased at 3.25 and again at 4.25). The liver apparently has little free glycogen to convert (sharp decline of sugar in lymph) and the first of the sharp leukocytic waves makes its appearance. Lymph protein and volume have reached a maximum, as has the temperature of the animal.

The usual premortal increase in the K/Ca ratio now becomes apparent with a final high ratio of 2.75; erepsin increases, lymph volume remains high, sugar diminishes, and with the peripheral dilatation (leukocytosis and the splanchnic exhaustion) the temperature diminishes. The urine flow ceases at the time of maximum injury. The marked fluctuations of the leukocyte count afford an interesting side light into the rapid changes in autonomic tonus. From this, as from other graphs it seems possible that these fluctuations may be correlated directly with the change in the K/Ca ratio. It will be noted that the four leukocyte peaks follow the four peaks of the K/Ca ratio (1.79, 2.12, 2.10 and 2.75).

At no time did colon bacilli come thro into the lymph stream, nor was there any appreciable alteration in the concentration of lymph agglutinins or precipitins for *B. coli*. A total of 600 cc. of suspension was injected.

² Petersen, W. F., and Müller, E. F.: Arch. Int. Med., 1927, 40, p. 575.

CHART 2

EXPERIMENT 51
CONTINUOUS INJECTION B.COLI
200 cc FROM 12:15 TO 3:15
400 cc FROM 3:25 TO 6:15
TOTAL 600 cc = 5 SEALS

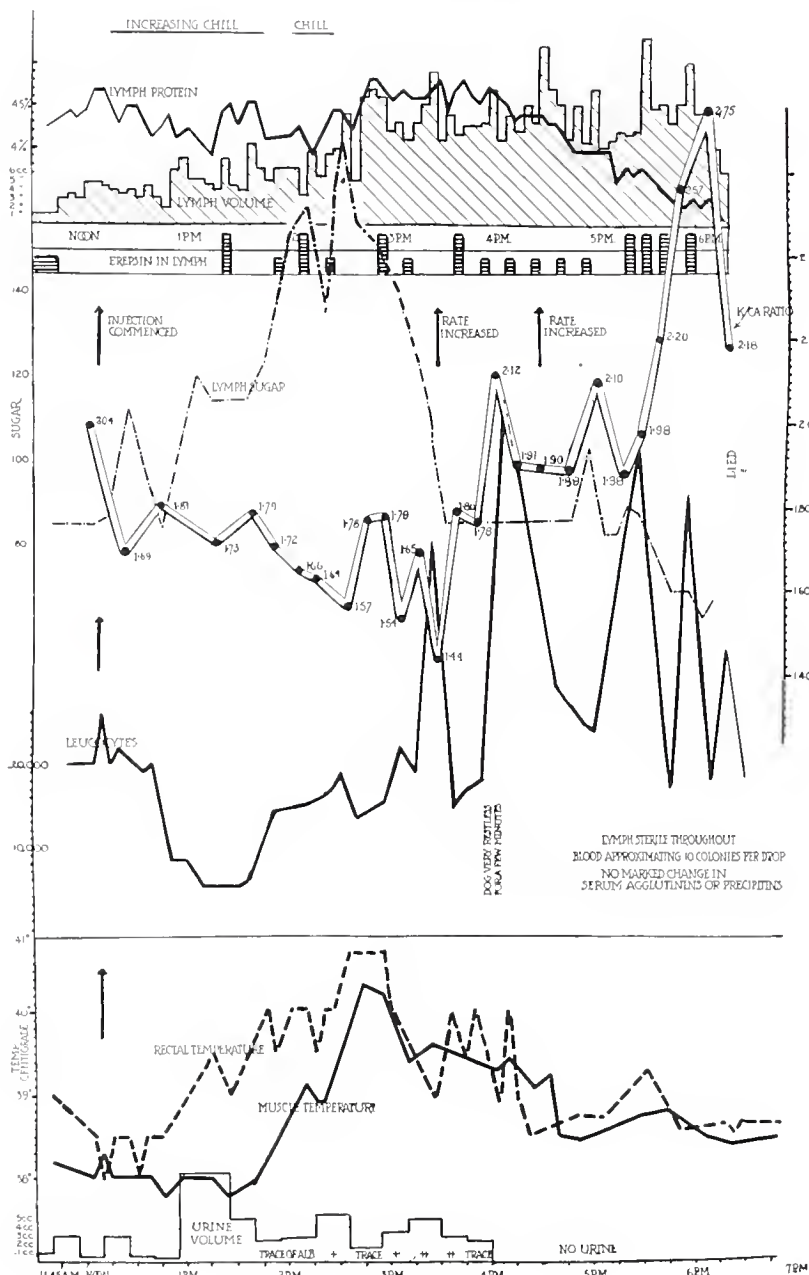
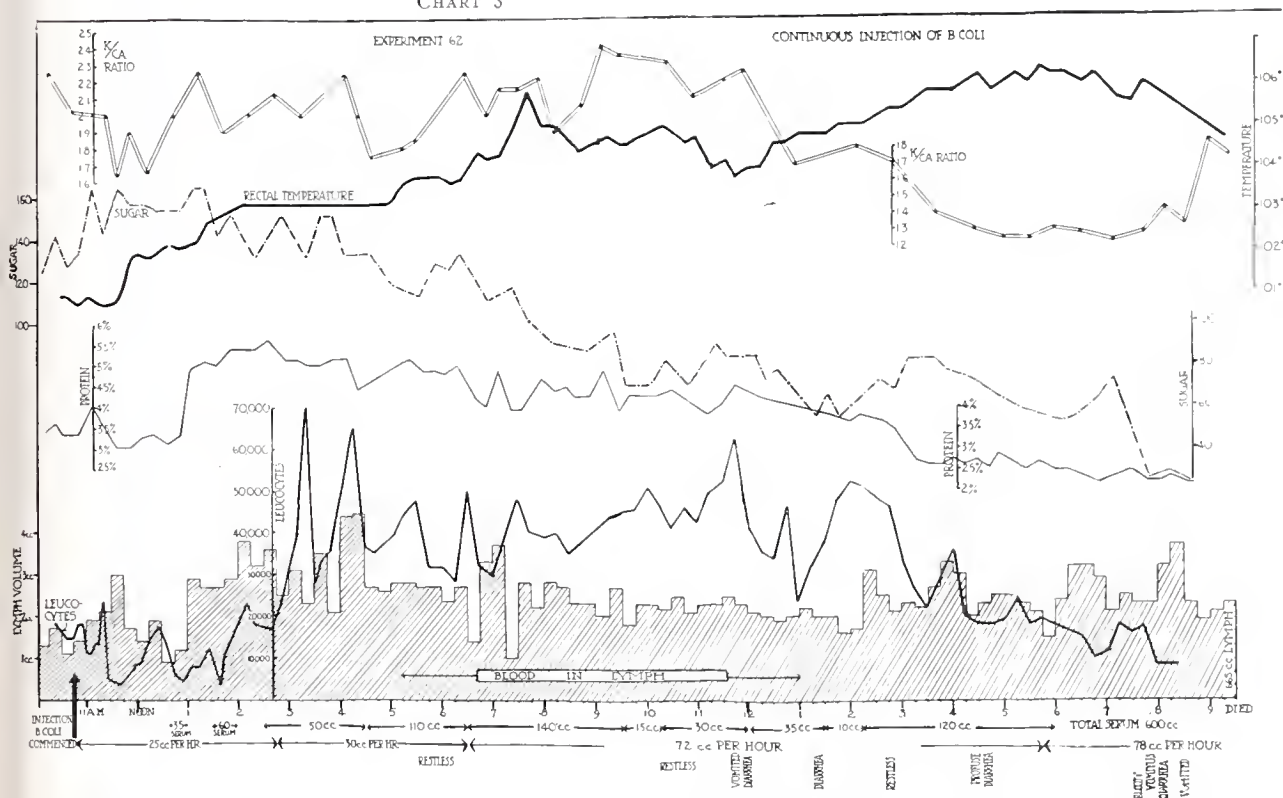


Chart 3.—A normal dog, weight 7.5 kilo. Continuous intravenous injection and in addition a total of 600 cc. of normal dogs serum was injected to replace the loss of fluid due to the prolonged lymph drainage. The total lymph collected was 665 cc. The injection (1 slant B. coli and 1000 cc.) was made at a slow rate, in order to permit the normal flow of injury and recovery in the equilibrium of the cells free play.

The normal control period was 45 minutes, with normal values established for the various curves. In analyzing the curves following the injection, they can roughly be divided into two parts, the period before midnight, and the period following.

CHART 3



In the period up to midnight, a constant wave like fluctuation was apparent in K/Ca ratio, leukocytes, lymph volume and protein and sugar. The first reaction of the injection is a reduction of the K/Ca ratio, sugar increase, protein increase and leukocyte fall. This lasts not more than 15 or 20 minutes and then definite waves are set up with the K/Ca ratio reaching a high level of 2.4 at 9 P. M. During this time the animal is not prostrated, but restless, the temperature while rising, does so very slowly, probably to be accounted for by the considerable periods of peripheral dilatation (leukocytosis). The sharp fluctuations in the leukocyte curve are usually synchronous with changes in the K/Ca ratio.

At midnight evidences of severe clinical injury became apparent. The animal vomits and has a severe diarrhoea, which persists at various intervals

until death. The K/Ca ratio sinks continuously for the next 7 hours, as do likewise sugar, lymph protein and leukocytes. During this time the animal is still capable of splanchnic stimulation, because the temperature increases, but this may in part be due to lessened peripheral heat loss (leukocytes diminished).

The terminal picture obtains from 7 o'clock until death at 9.30. During this time the K/Ca ratio increases sharply, the sugar sinks still more, and the temperature also begins to decline. Lymph cultures were sterile throughout.

In the next group of experiments we deal with animals that were apparently normal when selected, but in whom certain unusual preliminary chemical reactions were obtained.

Chart 4.—An apparently normal dog, weight 13 kilo. The control period is characterized by a high lymph sugar, lymph volume and protein concentration, by a leukocytosis and by a relatively high Ca content, with a ratio consequently slight below 2.

Injection is followed by an increase in the K/Ca ratio, a fall in the protein concentration of the lymph, preliminary leukocytosis and no great increase in lymph volume. It is obvious that the reaction in this dog, with its pre-injection relations abnormal, is directly the reverse of the normal.

Following this preliminary reaction the typical fall in the K/Ca ratio takes place and with it a leukopenia, a transient increase in protein, etc. Great fluctuations in the leukocyte curve continue.

Maximum injury apparently takes place some time between 10 and 11 P. M. (The rate of injection was increased at 11.20 P. M.). An erythema is first noted, with it a fall in lymph protein, then an increase in nonprotein nitrogen, and with that a steep rise in the K/Ca ratio (terminal). At this time bacteria pass through to the lymph for a short time.

The typical terminal curves then rapidly follow. Two slants of *B. coli* were injected from 2.45 till 11.20, and one slant from 11.20 to the time of exit.

Chart 5.—A presumably normal dog, weight 12 kilo. Control orientation similar to the preceeding, i. e., high lymph sugar, low K/Ca ratio, possibly due to rather prolonged operative manipulation of thoracic duct incannulation. During the control period of two hours, traction was made by accident on the vagus while inserting blood cannula for blood pressure determination. This was followed by a leukocytosis and by a considerable rise in lymph sugar. It will be noted that the lymph sugar level is considerably above that of the blood serum.

The injection was followed by an increase in the K/Ca ratio, a fall in lymph sugar, in lymph volume, and an increase in blood pressure.

Then as a secondary reaction a sharp fall in blood pressure, a rise in lymph sugar, in lymph volume and protein, leukopenia, etc., take place. At 1.10 the animal was moderately salivating. However, the K/Ca ratio does not fall until the end of the chill, i. e., at about 1.30 P. M.

The temperature reactions are characteristic. During the time of chill the rectal temperature has increased rapidly, but the muscles remain cold until the end of the chill approaches, and the synchronous leukocytosis gives evidence of a peripheral dilatation. That the dilated capillaries which account for the drop in the blood pressure are those of the splanchnic area is apparent from the fact that during the time of the drop the muscle capillaries are still contracted (leukopenia). The final rise in the blood pressure on the other hand, takes place even while the peripheral vessels show dilatation (leukocytosis).

CHART 4

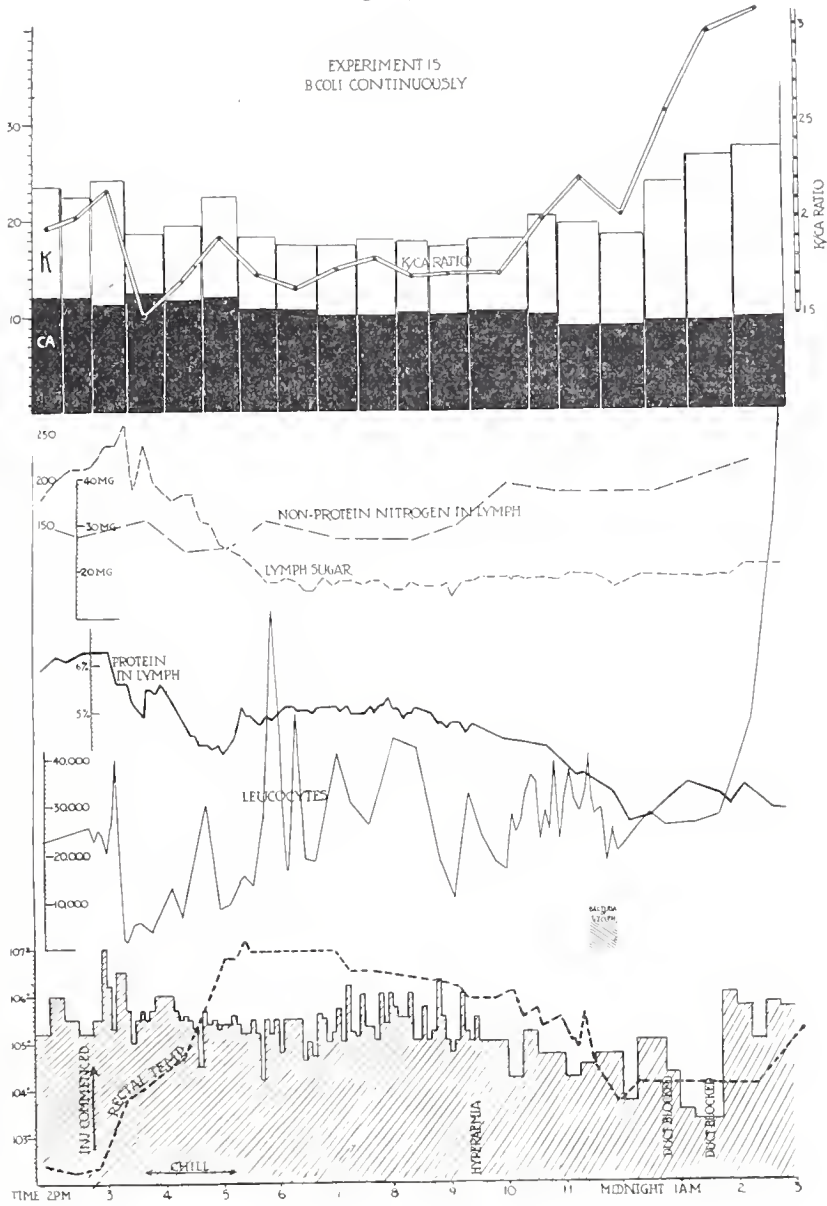


CHART 5

EXPERIMENT 20
CONTINUOUS INJECTION
OF B. COLI

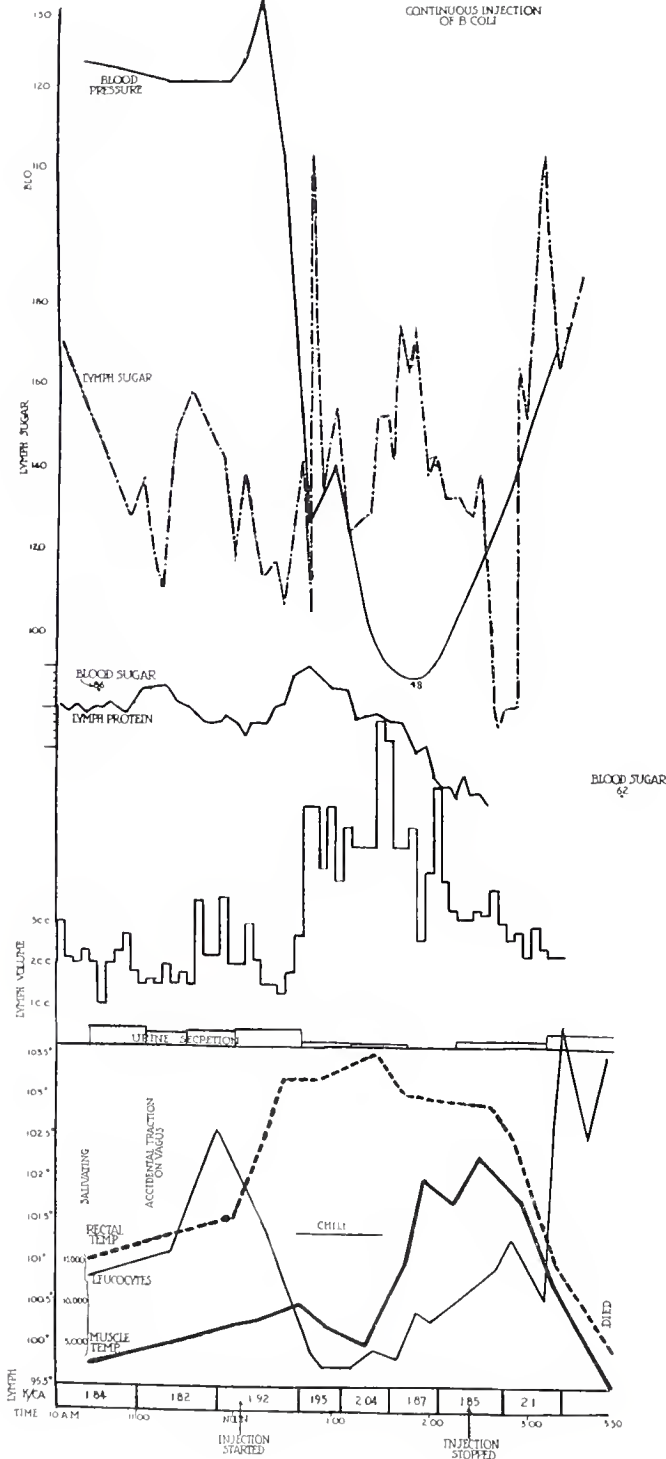
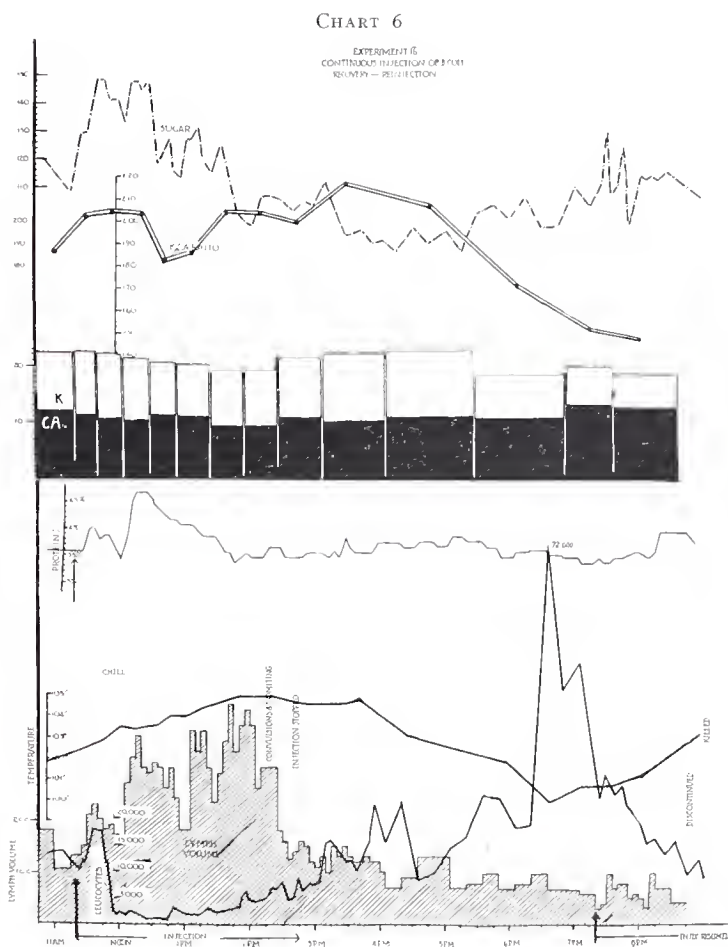


Chart 6.—A normal dog, weight 12 kilo. In this final experiment we present the effect of a discontinuous injection, but again in a dog in which the K/Ca ratio at the beginning of the experiment was below 2, with a relatively high Ca level of the lymph. The animal was starved 36 hours, but in other respects practically normal, as the control curves indicate.

Injection was followed by an increase in the K/Ca ratio (calcium diminishing), an increase in leukocytes, sugar, and a moderate increase in lymph volume and protein.



The usual reversal now takes place and with the sinking of the K/Ca ratio, leukopenia is established, and with it a marked increase in lymph volume and protein.

By 2.15 P. M. the animal was severely injured (vomiting and convulsions), this taking place when the calcium was again coming from the tissues and the temperature had reached a maximum. The injection was now discontinued.

Despite this discontinuance, the K/Ca ratio continued a steep and uninterrupted descent. A sharp leukocytosis occurred after 6.30 (with this the temperature declined, as would be expected with peripheral dilatation).

Injection was recommenced, but the animal finally killed at 8.45. The terminal increase in potassium and K/Ca ratio had not been reached at this time.

During the initial period 170 cc. were injected, and 75 cc. during the final period.

DISCUSSION

When *B. coli*, which we have used as a typical endotoxic organism, are slowly and continuously injected intravenously in dogs, a series of clinical reactions take place which are typical for the onset of any acute infection, and the reactions which supervene can roughly be divided into 5 periods: the latent period; the period of splanchnic stimulation; the period of recovery, with a series of fluctuating phases following; the period of maximum injury; and finally, the premortal period.

The experiments make apparent a number of rather interesting correlations:

During the initial latent period the bacteria injected into the blood stream produce no marked changes. They are, however, immediately fixed by the reticulo endothelial system (largely in the liver), and when so fixed begin to stimulate the tissues.

This initiates the second phase. During this time the splanchnic vasodilatation and splanchnic leukocytosis takes place. Lymphorrhagia occurs, together with increased concentration of protein in the lymph, sugar mobilization, reduction in lymph coagulability, the appearance of soluble liver proteins in the blood stream (Andrews and Thomas), and increased phagocytosis by the reticulo-endothelium (Jaffé). We have used the term³ "parasympathetic status" for this phase of the splanchnic activity, and as a result of this autonomic fixation the peripheral regions are oppositely oriented (sympathetic) with the diminished activity, the chill and leukopenia being the expression of this change at the periphery. The increase in temperature is the result of the splanchnic stimulation and the peripheral reduction of radiation. The chemistry of the lymph reveals these changes very well. A lowering of the K/Ca ratio takes place, as well as the lowering of the CO₂ combining power. After this initial period of stimulation the third phase makes its appearance.

The cells make an effort to restore the disturbed balance and with it definite wave-like fluctuations are to be observed in all curves. These

³ Müller, E. F., and Petersen, W. F.: *Klin. Wchnschr.*, 1926, 5, p. 53.

waves seem to be definitely correlated in the case of leukocytes, the K/Ca ratio, the lymph sugar and the lymph proteins.

Then apparently the maximum injury occurs. The time of this occurrence varies in different animals, with the concentration of the bacteria, with the supply of glycogen of the liver, etc. It is during this time that a few bacteria may be swept from the blood stream to the lymph stream. At all other times the fixation of bacteria by the reticulo-endothelial cells seems to be absolute. The efficacy of the reticulo-endothelial system, despite the continuous intoxication of the animal, seems to be remarkable, when we consider that from 5 to 10 million organisms are being injected per minute over long periods of time.

The period of maximum injury is associated with a greater liberation of the erepsin, by the increase in the nonprotein nitrogen, by the progressive fall in the K/Ca ratio, by glycogen exhaustion of the liver, by leukocytosis, by the maximum lymphagogue effect; and clinically by a fall in the temperature, by erythema, persistent nausea and vomiting, and occasionally by convulsions.

Finally the premortal period can be recognized. With this the K/Ca ratio increases, due very likely to a liberation of potassium from injured red blood corpuscles. Lymph production and protein diminish, as does lymph sugar and CO_2 combining power. The appearance of hemoglobin, bile and red blood corpuscles in the lymph (which may have commenced sometime during the third period) reaches its maximum, and there is usually a terminal leukocytosis of considerable extent.

While this is the usual sequence of events, the preliminary reaction may occasionally be different. In animals with a low K/Ca ratio (these usually with increased lymph flow, with increased lymph protein and sugar, and a leukocytosis) we have observed that the primary reaction to the injection of the bacteria will be characterized by an increase in the K/Ca ratio, and with it a leukocytosis, a reduction in the lymph protein, occasionally of the sugar, and the lymph volume. Such paradoxical reactions are similar in character to those frequently observed with pharmacological agents, and evidently depend on an altered chemical status of the tissue cells and fluids.

SUMMARY

The clinical reaction of dogs to the continuous injection of B. coli has been studied and correlated with simultaneous lymph analysis. The reaction can be divided into five periods: the latent period; the

period of splanchnic stimulation; the period of fluctuations, with cellular efforts of restoration of the equilibrium; the period of maximum injury, and the premortal period. Only during the period of maximum injury do bacteria pass from the blood stream to the lymph channels. In certain animals (differing chemically from the normal) the preliminary reaction may be reversed (paradoxical reactions).

THE IMMUNOLOGIC RELATIONS OF TYPE 4 PNEUMOCOCCI OBTAINED DURING AN EPIDEMIC

G. H. ROBINSON

From the William H. Singer Memorial Research Laboratory and the Allegheny General Hospital, Pittsburgh, Pa.

Since the biological classification of pneumococci into types 1, 2, 3, and 4 by the various workers at the Rockefeller Institute, type 4 has generally been regarded as a miscellaneous group of pneumococcus-like organisms containing no important fixed immunologic subgroups. There has been no question of the pathogenicity of some type 4 organisms as they have been found in all conditions such as are produced by the fixed types 1, 2, and 3. However, there is an important problem in the relation of those strains found in normal healthy individuals to those having definite disease-producing powers. This report includes a study of the immunologic relations of strains of type 4 pneumococci obtained during a season in which this organism predominated. The distribution of the groups is traced throughout this period and the relative virulence of the strains, as evidenced by the outcome of the human infection is noted. The subject matter is unique in that, so far as I am aware it is the first laboratory study of an epidemic of type 4 pneumonia. The high incidence of pneumonia in Pittsburgh offers an important public health problem. Its solution must involve a study of a great many factors. Of these a thorough study of the etiologic agent and especially the rôle of type 4 pneumococcus is of primary importance.

The relative occurrence of type 4 pneumonia may be seen from the tabulation of its incidence and mortality as found in different cities.

	Incidence	Mortality
	%	%
New York ¹	24	16
Chicago ²	28	25.8
Baltimore ³	25.9	57.2
Philadelphia ⁴	38	33
Pittsburgh ⁵	55.7	—*

* McMeans did not collect the mortality statistics from type 4 pneumonia in Pittsburgh.

Received for publication, Oct. 13, 1927.

¹ Monographs of the Rockefeller Institute for Medical Research, No. 7, 1917, p. 33.

² Mathers, G.: J. Infect. Dis., 1915, 17, p. 514.

³ Clough, M. C.: Bull. Johns Hopkins Hospital, 1917, 28, p. 306.

⁴ Lukens, F. D. W.: Bull. of the Ayer Clin. Lab. of the Pennsylvania Hospital, No. 10, 1926, p. 20.

⁵ McMeans, J. W.: Atlantic M. J., 1927, 30, p. 499.

The statistics for the first four cities include cases occurring during a year or more. McMeans' statistics, compiled from the laboratory records of five Pittsburgh hospitals, give the average for the last nine years. The annual proportion of type 4 pneumonia in Pittsburgh has varied from 40% to about 70% of all pneumonias, being, therefore, between two and three times as prevalent as in some of the other cities of which we have records. On the other hand, type 1 is considerably less common in Pittsburgh than in other cities, as can be seen by comparing McMeans' 15% with three series from New York City showing 35%, 34%, and 33%, and three series from Philadelphia showing 31%, 37%, and 29% (Lukens' ⁴ table 3). Thus it appears that over a series of years there has been in Pittsburgh an unusual virulence of, or a peculiar susceptibility to type 4 pneumococcus.

While various epidemiological studies indicate that types 1, 2, and 3 are the important predominant types in the United States, Lister's ⁶ work in South Africa shows that the same conditions may not prevail uniformly throughout the world. Although one-third of the strains which he obtained belonged to these fixed types, the predominant organism, recovered from 39% of his cultures was either another fixed type or a subgroup of type 4. He also noted a variation in the relative occurrence of the different types from year to year.

Several attempts have been made to classify strains of type 4 pneumococcus. Cooper, Mishulow, and Blanc ⁷ have studied 55 strains from normal individuals and from those having various infections of the respiratory tract, including influenza. They found no predominating type and no correlation between the condition of source and the organism obtained. Olmstead, ⁸ from a study of 94 strains decided that type 4 pneumococci are classifiable. She found 12 definite groups, in only 3 of which did the strains from pneumonia exceed 50% of the members of that group. Forty-six of her strains were obtained from the sputum of normal persons. Gordon, ⁹ investigating the pneumococci from normal throats, common colds, and other respiratory infections, found that the type 4 strains from colds and influenza were likely to be more virulent than those from normal individuals and they also showed a tendency towards grouping. He also obtained the same serologic group of type 4 from persons suffering an epidemic of tonsillitis and pharyngitis.

⁶ South African Institute for Medical Research, Publication 8, 1916.

⁷ J. Immunol., 1921, 6, p. 25.

⁸ Ibid., 1917, 2, p. 425.

⁹ J. Infect. Dis., 1921, 29, p. 437.

Experimental Work.—During the winter season of 1925-26 rabbits were immunized against a few type 4 strains, but, as there was no apparent immunologic relation between them, the work was discontinued. There were no type 4 pneumonias in the hospital during the first few months of 1926. At that time there was an unusually large number of type 3 cases (table 1). A comparison of the types of pneumococcal infections in January, 1927, however, indicated an abnormal predominance of type 4 which amounted to a type 4 epidemic (table 1). Again our interest was focused on the immunologic relations of these organisms. The cultures used in the following work were obtained during the epidemic.

Sputum from hospital patients having clinical symptoms of pneumonia was treated according to the standard method for typing by the mouse test. Cul-

TABLE 1
INCIDENCE OF INFECTION AND MORTALITY FOR TYPES OF PNEUMOCOCCI IN THE ALLEGHENY
GENERAL HOSPITAL

	Types	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total
1926	1	1	1	2	3	0	0	0	0	1	0	1	0	9 (no deaths)
	2	0	0	0	2	5 (1)	3	2 (1)	1	0	2 (1)	2 (1)	4	21 (4, 19%)
	3	2	6 (4)	14 (7)	8 (4)	3 (2)	2	1	2 (1)	0	0	1 (1)	2	41 (19, 46.3%)
	4	0	0	0	0	4	3 (1)	1	1	5 (2)	6 (2)	2	5	27 (5, 18.5%)
	Types	Jan.	Feb.	Mar.	Apr.	May	June	Total						
1927	1	0	2	3	1	1	0	7 (no deaths)						
	2	2	2 (2)	2	1	0	1	8 (2, 25%)						
	3	0	2 (1)	0	1	0	0	3 (1, 33.3%)						
	4	11 (5)	9 (2)	10 (4)	15 (5)	8 (3)	6 (3)	59 (22, 37.3%)						

* Figures in parentheses indicate number of deaths.

tures were made from the heart blood of the mouse in order to preserve the strain if it should prove not to be a fixed type. Type 4 strains were tested for bile solubility and the fermentation of inulin.

Rabbits were immunized by giving intravenous injections at three or four day intervals. The usual procedure was as follows: one-half the growth on a blood agar slant, killed by exposure to 60 C. for 15 minutes was given for each of the first two injections; one whole agar slant, 7 days old, for the third injection; the 24-hour growth on an agar slant for the subsequent doses. The rabbits withstood this treatment well, although with some strains the 24-hour growth had to be given in smaller doses. As a rule, blood taken 4 days after the fourth injection showed strong agglutinative power for the homologous strain in a dilution of 1:2.

For the agglutination tests the 24-hour growth of pneumococci on a blood agar slant was washed off with salt solution and made up to a suitable turbidity. Serum and suspension were mixed in equal parts. After trying incubation at 55 C., at 37 C., and at room temperature it seemed that the best reactions were obtained at the lowest temperature. In fact, if the serum was potent enough, agglutination took place almost instantly and there were no partial reactions observed. Final readings were made after the tests had remained at room temperature over night.

Obviously the selection of strains for immunization was done at random and the sequence of groups was in the order of their establishment. Not all strains could be recovered from the heart blood of the mice and several strains were lost before they could be tested against all of the immune serums. For that reason it has seemed more illuminating to express the percentages grouped of the total number tested during the different months (table 2).

Up to the present 65 strains of type 4 pneumococci from pneumonia patients have been studied besides a few strains from tuberculous and normal sputum. Not all of them have been grouped as yet, although they have all been tested against 12 distinct immune serums. There have been 8 immunological groups demonstrated. Groups 4a, 4c, and 4d contain 7 strains each; group 4b includes 6 strains; and the other groups consist of 2 or 3 strains each. All of the strains from nonpneumonic sources are included in the smaller groups. Groups 4a, 4b, and 4c occurred uniformly during the early months of the epidemic and for that reason their incidence is combined in table 2. Group 4d, however, did

TABLE 2
RELATIVE INCIDENCE OF GROUPS OF TYPE 4 PNEUMOCOCCUS EXPRESSED IN PERCENTAGE OF STRAINS TESTED

	Dec.	Jan.	Feb.	Mar.	Apr.	May	June
Groups 4a, 4b, 4c.....	75	67	50	50	7	20	0
Group 4d.....	0	0	0	20	20	20	33

not appear until March and then predominated. The mortality of patients infected with the different groups was 14% in group 4a, 33% in group 4b, 56% in group 4c, and 28% in group 4d. There were two deaths among the other four groups. Thus strains from 11 of the 19 cases terminating fatally fell into 6 of the 8 groups. The virulence of group 4c is further substantiated by the fact that 4 of the 7 strains were obtained from blood cultures.

It was thought that perhaps the serum of convalescent pneumonia patients might serve for identifying immunologic groups. No conclusive results could be obtained with this serum in the protection of mice against infection. The serum did not always show agglutinins and when present, they were not as potent as could be produced in rabbit serum.

DISCUSSION

A comparison of the figures for 1926 with those for 1927 shows that there was a disproportionately large number of type 4 pneumonia infections in the early part of 1927 resembling an epidemic. While the total number of cases is comparatively small, they most probably typify the

condition in Pittsburgh as a whole. An analysis of the immunologic relations of type 4 strains obtained during this epidemic indicates that they are not only capable of being grouped but that the epidemic was due to a few definite subgroups.

During the first month of type 4 predominance, 75% of the strains tested fell into 3 distinct groups. During the next three months 67%, 50% and 50% respectively were found to be of the same group. In April a marked change was noted in the bacterial flora of the sputum. Only 7% of the strains could be placed in the 3 groups which had previously predominated, a new group of pneumococci appeared and Pfeiffer's bacillus occurred in large numbers, in fact, so numerous that it was difficult to isolate these pneumococci. The majority of the pneumococci obtained since April have been immunologically heterogeneous, in that respect, I believe, resembling the type 4 strains obtained from normal sputum.

The mortality rate for 1926 among type 4 infections had been 18.5%. During the six months of type 4 ascendancy the mortality rate was 37.3%, indicating an increased virulence as well as incidence. The average mortality (33%) among the patients from whom strains were grouped is approximately that for the whole period. Therefore, these groups were apparently typical of the whole epidemic. The increase in the death rate for 1927 is due principally to the deaths of those infected with group 4c (56%). If these are excluded the mortality among those infected with the other 3 groups would be 25% or slightly more than for the nonepidemic period of 1926.

Those who have studied type 4 pneumococci from normal individuals have found a large number of immunologic groups, each containing a few strains. It is possible that under some conditions these organisms may give rise to respiratory infections. In the epidemic under discussion the etiologic factor was originally three groups of virulent type 4 pneumococci. A few strains of the usual type 4 pneumococci were obtained at this time. Then the type of respiratory tract flora changed until practically all of the pneumococci obtained were of the usual (non-pathogenic?) types. The cause of this change is a field for speculation.

The occasional selection of the heterogeneous strains of pneumococci commonly found in normal sputum seems inevitable when the mouse test is employed. In three cases of seven in which both sputum and blood cultures were tested, the sputum culture was heterogeneous while the blood culture belonged to one of the predominating groups. Hetero-

geneous strains may be primary infecting agents in sporadic cases of type 4 pneumonia and some may assume sufficient virulence to produce epidemics. On the other hand there are probably "fixed types" not as yet described. It is quite possible that certain of these uncommon "fixed types" may be peculiar to Pittsburgh.

CONCLUSIONS

Strains of type 4 pneumococci obtained during an epidemic could be resolved into a number of immunologic groups varying in their relative virulence toward man. During this period of the excessive incidence of type 4 the infection was caused by a few type 4 subgroups. The evidence indicated that epidemic strains could be classified while those from the sputum of normal individuals are probably more nearly heterogeneous. The epidemic groups obtained may be rare fixed types peculiar to certain localities. Normal sputum strains are sometimes obtained by the mouse test while the strains causing the infection in the patient is entirely missed.

STREPTOCOCCI AS A CAUSE OF SPONTANEOUS ABORTION

ALLAN F. REITH

From the Department of Preventive Medicine, A. O. Smith Corporation, Milwaukee, Wisconsin

It is generally estimated ¹ that one out of every five or six pregnancies terminates in abortion. Many of these abortions are caused by defective germ plasm or intrinsic disturbances in the fertilized ovum, by pelvic malformation, dietary deficiency, trauma, or induction,² but many others, the so-called idiopathic abortions, cannot be explained by these causes.³ Acute chronic infections have been mentioned by several observers ⁴ as causes for abortion, but only a few reports on experimental work have been recorded. Curtis,⁵ in 1916, injected five pregnant rabbits with two cultures of streptococci, one from the urine of a mother whose child was born dead, and one from the heart blood of a stillborn child, with the result that abortion or absorption of the fetuses took place in each of the rabbits. Pure cultures of streptococci were recovered from each uterus. More recently he reported ⁶ on intravenous injections, in seven pregnant rabbits, of broth cultures of hemolytic streptococci obtained from the urine of a woman who had had eleven spontaneous abortions. Abortion or absorption of the fetuses took place in every rabbit. To note the influence of nonspecific infections on the course of pregnancy he injected six pregnant rabbits intravenously with virulent colon bacilli. If the dose was the same as that of his first series, or less than that required to endanger life, there was no interruption of pregnancy. The presence of streptococci in the urine of this woman he attributed to infected teeth.

Browne and Kincaid ⁷ gave injections intravenously in four rabbits with hemolytic streptococci isolated from the heart blood of a macerated full term fetus. One rabbit aborted, and in two there was fetal death

Received for publication, Oct. 13, 1927.

¹ Williams, J. W.: *Obstetrics*, 1920, p. 661. Rock, J.: *Boston M. & S. J.*, 1926, 195, p. 843. Curtis, A. H.: *J. Am. M. A.*, 1925, 84, p. 1262. Slemmons, J. M.: *The Prospective Mother*, 1922, p. 170.

² Macomber, D.: *Boston M. & S. J.*, 1925, 193, p. 116. Rock, J.: *Ibid.*, 1926, 195, p. 843.

³ Browne, W. H., and Kincaid, H. L.: *J. Am. M. A.*, 1926, 87, p. 847. Williams, J. W.: *J. Obst. & Gynecol. Brit. Empire*, 1925, 32, p. 259.

⁴ De Lee, J. B.: *J. Am. M. A.*, 1916, 67, p. 344. Curtis, A. H.: *Ibid.*, p. 1739. Talbot, J. E.: *Boston M. & S. J.*, 1922, 187, p. 315.

⁵ *J. Am. M. A.*, 1916, 67, p. 1739.

⁶ *Ibid.*, 1925, 84, p. 1262.

⁷ *Ibid.*, 1926, 87, p. 847.

with partial absorption; hemolytic streptococci were recovered from the amniotic fluid or the fetal heart blood. The fourth rabbit, given a small dose, remained normal. They concluded that the fetus of their patient had died of hematogenous streptococcic infection.

I have had opportunity to conduct similar experiments on animals, with streptococci obtained from the tonsils and a placenta of a woman who had had five spontaneous abortions. Her general physical condition was excellent. The routine laboratory tests of the blood and urine were negative. A blood culture by special method⁸ yielded green-producing, anaerobic, pleomorphic streptococci. Her tonsils were buried and red-dened, and culture from a swab gave a scanty growth of pleomorphic, faintly hemolytic streptococci on blood agar plates. In 0.2% glucose beef heart medium these organisms grew profusely in the lower half of the tube, and poorly in the upper half. In the experiments recorded in this paper all rabbits were given injections intravenously with 18 to 20-hour cultures in 0.2% glucose beef heart medium, using 3 cc. per kilogram of body weight.

Four female rabbits were given injections with cultures from the tonsils. Two rabbits (1 and 2) aborted within 48 hours; rabbit 1 died on the third day; rabbit 2 was killed on the fourth day, and when examined it was noted that one horn of the uterus had emptied, while five dead fetuses, partially macerated, were found in the other horn. Two other rabbits (3 and 4) were not pregnant; rabbit 3 died within 24 hours, and rabbit 4 was killed on the fourth day after injection. Hemorrhagic areas were found in the uterine wall of each of the four rabbits, and no gross lesions were observed in the other organs. Pleomorphic, faintly hemolytic streptococci were obtained in pure culture from the heart blood and from washings of the uterus of each rabbit. The organisms grew scantily, or not at all, on blood agar plates, but abundantly in tubes of glucose beef heart medium, from which transfers to blood agar plates resulted in fair surface growth.

Two more rabbits (5 and 6), received injections with pure cultures of streptococci from washings of the uteri of rabbits 1 and 2, respectively. Both died during the following day: in each the uterus was hemorrhagic, and streptococci were recovered from it. The same result was obtained in one of two rabbits into which were injected cultures from washings of the uterus of rabbit 6, while the uterus of the other rabbit remained normal.

⁸ Reith, A. F.: *J. Bact.*, 1926, 12, p. 367.

During these experiments the patient's tonsils were removed. About one month later she again aborted, and the placenta was obtained under relatively aseptic conditions. No evidence of syphilis was found by the pathologist. A portion of the placenta was thoroughly seared, and a piece, weighing about one gram, was excised with sterile instruments. Glucose beef heart cultures of this piece of placenta, of surface swabs, and of the bloody exudate all yielded pleomorphic diplococci or short-chained, pleomorphic streptococci, which did not grow on blood agar plates from the first generation.

Three female rabbits were given injections with cultures from the placenta. Two of them (7 and 8) aborted on the following day. The third (rabbit 9) which was nonpregnant, died within 24 hours, and extensive hemorrhages were found in the uterus. Anaerobic, pleomorphic streptococci were recovered from the uterus of each rabbit and from the liver of one aborted fetus. Two control rabbits (10 and 11) were given injections at the same time with cultures of streptococci from the apex of an extracted tooth. Both were killed the following day. The uterus of each rabbit was normal and cultures were negative for streptococci.

Three nonpregnant rabbits were given injections with cultures of washings from the uterus of rabbit 9. Two developed hemorrhage in the uterus, and from the uterus of the third rabbit, although no hemorrhage was observed, streptococci were recovered. Diplococci and short-chained streptococci were found in several sections of the uteri from these rabbits.

A throat culture from the patient ten months after tonsillectomy gave a scanty growth of green-producing streptococci on blood agar plates. Short-chained streptococci were seen in smears from cultures in glucose beef heart medium. A blood culture made by the method previously mentioned was negative. Six female rabbits which received injections with this throat culture did not appear sick and had lost no weight at the end of five days, when they were killed and examined. All were nonpregnant, and no hemorrhages in the uterus were found on gross or microscopic examination, nor were streptococci recovered in culture from any of the uteri.

As a further control 7 female rabbits were given intravenous injections with cultures of the prostatic secretion and of swabbings from the tonsil of the patient's husband. The culture of the prostate contained anaerobic, anhemolytic streptococci, and the culture of the tonsil green-producing streptococci. The rabbits lost slightly in weight, but did not appear sick after four to five days, when they were killed and examined.

Two of the rabbits were pregnant, and when killed the fetuses were living and apparently normal. The uterus of each of the other rabbits appeared normal, and streptococci were not recovered in culture.

DISCUSSION

During the course of these experiments in this laboratory 167 female rabbits have been given intravenous injections of glucose beef heart cultures of streptococci recovered from various foci of infection. As a rule the rabbits used were young adults weighing from 1200 to 2000 grams. Nineteen had hemorrhages in the uterus or aborted after the injection, and of this number 17 had been injected with cultures from women who had had spontaneous abortion. Only two (1.3%) of the rabbits which received control cultures from other persons showed lesions in the uterus (one hemorrhage, one abortion). Fifteen rabbits were pregnant at the time of injection. Four of these were given injections with cultures from the woman mentioned in this report, and all four aborted, whereas pregnancy was not interrupted in ten rabbits given cultures from persons who had not had abortions. Pregnancy was interrupted in only one other rabbit, which received the culture of a tonsil from a man who had chronic appendicitis.

These observations suggest that the streptococci with which I was dealing possessed special properties for invading the uterus. It is interesting to observe that the organisms were pleomorphic, green-producing or faintly hemolytic streptococci which grew best under reduced oxygen tension, and had the cultural characteristics which were also found in the streptococci described by Rosenow⁹ in his extensive work on "elective localization" of bacteria from foci of infection.

De Lee¹⁰ and Macomber¹¹ believed that focal infection, by setting up an endometritis with a strong tendency to become hemorrhagic, may be a cause of abortion. This opinion seems to be confirmed by my experiments in which 8 out of 10 nonpregnant rabbits given injections with cultures of tonsil or placental origin developed hemorrhages in the uterus.

SUMMARY

Pleomorphic, anaerobic, green-producing or faintly hemolytic streptococci from the tonsils and placenta of a woman who had had repeated spontaneous abortions produced abortion in four pregnant rabbits and

⁹ J. Dental Res., 1919, 1, p. 205.

¹⁰ The Principles and Practice of Obstetrics, 1924, p. 445.

¹¹ Boston M. & S. J., 1925, 193, p. 116.

hemorrhage in the uterus in 8 out of 10 nonpregnant rabbits after intravenous injection. In 13 rabbits given injections with a throat culture from the same woman 10 months after tonsillectomy, and with tonsil and prostate cultures from her husband, no lesion in the uterus were seen. These results corroborate the theory that foci of infection may harbor streptococci which possess properties that may give rise to spontaneous abortion.

THE DURATION OF IMMUNITY TO *B. WELCHII* TOXIN IN RABBITS

GUILFORD B. REED AND MARION SPENCE

From the Department of Bacteriology, Queen's University, Kingston, Canada

Recent papers by Cornell¹ and from this laboratory² have shown that *B. welchii* produces long standing infections in rabbits, accompanied by an anemia which resembles pernicious anemia in man. Kahn and Torry³ produced similar changes in monkeys with *B. welchii* toxin. From a quantitative study of the type of anemia produced in rabbits by *B. welchii* toxin, by tetanolysin, streptolysin and pneumolysin, we have already shown² that while similar degrees of anemia, as measured by decrease in circulating red cells and in hemoglobin percentage, may be produced by varying the dosage of these four hemotoxins only *B. welchii* toxin produces a measurable anisocytosis. It was also shown (Reed and Orr)² that in the in vitro hemolysis by these four toxins only *B. welchii* produces a definite change in the structure of the red cells which resist hemolysis.

Kahn and Torry³ found that monkeys developed an immunity to *B. welchii* toxin so that progressive doses became less effective and finally ceased to produce a reaction. Similar reactions might be expected from *B. welchii* toxemia in other animals including man. The recurrence then of toxemia from a reinoculation with toxin or from a quiescent infection will depend on the duration of the active antitoxic immunity. The present paper presents a limited amount of experimental evidence on the duration of this immunity in rabbits immunized by repeated injections of toxin.

Procedure.—The *B. welchii* toxins used in these experiments were prepared from several strains of organisms and were the same as those used in the experiments recorded in our last two papers.² The toxin was administered to normal rabbits in doses sufficient to give a definite anemia. As soon as the animal had recovered, as shown by blood count, hemoglobin percentage and the structure of the red cells, a second dose was administered and following

Received for publication, Aug. 4, 1927.

¹ Canadian M. A. J., 1925, 15, p. 26; J. Infect. Dis., 1925, 36, pp. 425, 508.

² Reed, G. B.; Orr, J. H., and Burleigh, C. H.: Canadian M. A. J., 1926, 16, p. 525. Reed, G. B.; Orr, J. H., and Spence, M.: J. Infect. Dis., 1927, 41, p. 283. Reed, G. B., and Orr, J. H.: 1927, 41, p. 289.

³ Proc. Soc. Exper. Biol. & Med., 1925, 23, p. 8.

recovery a third dose was given. After an interval of one to seven months the duration of the immunity was tested by determining the reaction to reinoculation with the same toxin used in the initial inoculations. The four case reports summarized in the tables are characteristic of the results obtained with several animals.

TABLE 1
PROGRESS OF IMMUNITY TO B. WELCHII TOXIN IN 4 RABBITS

Rabbit	Procedure	Date	Hemo-		Red Cell Structure
			Red Cell Number	globin %	
1	Normal.....		5,840,000	74	Normal
	Immunizing dose 1				
	1 cc. toxin	Sept. 29			
		2 hours later...	3,560,000	58	About $\frac{3}{4}$ microcytes
		6 hours later...	3,190,000	47	
		Sept. 30.....	2,170,000	37	Microcytes, macrocytes, poikilocytes
		Oct. 1.....	2,000,000	32	
		Oct. 2.....	2,500,000	27	Larger macrocytes
		Oct. 9.....	3,780,000	56	Many macrocytes
		Oct. 11.....	4,600,000	58	Macrocytes and normal
	Immunizing dose 2				
	1 cc. toxin	Oct. 12			
		6 hours later...	4,040,000	50	Many microcytes
		Oct. 13.....	4,110,000	56	
		Oct. 15.....	4,910,000	49	Normal and few microcytes, macrocytes
		Oct. 26.....	4,420,000	57	Mostly normal
	Immunizing dose 3				
	1 cc. toxin	Oct. 26			
		6 hours later...	4,410,000	67	
		Oct. 27.....	5,120,000	67	Normal
	Immunity testing dose				
	1 cc. toxin	Dec. 12 (7 weeks after dose 3)			
		6 hours later...	5,610,000	66	Normal
		Dec. 13.....	6,050,000	68	Normal
		Dec. 15.....	6,150,000	68	Normal
2	Normal.....		5,200,000	70	Normal
	Immunizing dose 1				
	0.5 cc. toxin	Dec. 3			
		2 hours later...	4,350,000	49	Many microcytes
		6 hours later...	4,360,000	48	Many microcytes
		Dec. 4.....	3,470,000	43	
		Dec. 5.....	2,940,000	40	Few microcytes, many macrocytes and poikilocytes
		Dec. 17.....	4,680,000	67	Normal
	Immunizing dose 2				
	0.5 cc. toxin	Dec. 18			
		2 hours later...	4,210,000	67	Many microcytes
		6 hours later...	3,260,000	59	Many microcytes
		Dec. 19.....	2,880,000	46	Microcytes and macrocytes
		Jan. 6.....	5,390,000	73	Normal
	Immunizing dose 3				
	0.5 cc. toxin	Jan. 7			
		2 hours later...	5,560,000	71	Normal
		6 hours later...	5,210,000	70	Normal
		Jan. 9.....	4,920,000	72	Normal
		May 30.....	5,500,000	79	Normal
	Immunity testing dose				
	0.5 cc. toxin	May 30 (4½ months after dose 3)			
		6 hours later...	5,600,000	64	Normal
		May 31.....	5,040,000	72	Normal
		June 3.....	5,030,000	67	Normal
		June 13.....	5,290,000	64	Normal

TABLE 1—Continued
PROGRESS OF IMMUNITY TO B. WELCHII TOXIN IN 4 RABBITS

Rab- bit	Procedure	Date	Red Cell Number	Hemo- globin %	Red Cell Structure
3	Normal.....		7,250,000	90	Normal
	Immunizing dose 1 0.5 cc. toxin	Dec. 13			
		6 hours later...	5,300,000	72	Many microcytes
		Dec. 14.....	5,120,000	70	
		Dec. 15.....	5,330,000	78	Mostly normal
	Immunizing dose 2 1.0 cc. toxin	Dec. 15			
		6 hours later...	4,130,000	48	Many microcytes
		Dec. 17.....	2,500,000	39	Few microcytes, many macrocytes
		Dec. 18.....	1,410,000	23	Mostly macrocytes, many poikilo- cytes, few nucleated red cells
		Dec. 21.....	2,020,000	29	
		Jan. 6.....	5,400,000	70	Normal
	Immunizing dose 3 1.0 cc. toxin	Jan. 7			
		6 hours later...	6,040,000	74	Normal
		Jan. 8.....	5,980,000	76	Normal
	Immunity testing dose 1.0 cc. toxin	May 30 (4½ months after dose 3)			
		6 hours later...	5,570,000	64	Many microcytes
		May 31.....	5,530,000	64	Many microcytes
		June 1.....	4,700,000	57	Many macrocytes
		June 3.....	4,450,000	57	
		June 14.....	3,680,000	56	Many macrocytes

PROGRESS OF IMMUNITY TO B. WELCHII TOXIN IN 4 RABBITS—CONTINUED

Rab- bit	Procedure	Date	Red Cell Number	Hemo- globin %	Red Cell Structure
4	Normal.....		6,000,000	88	Normal
	Immunizing dose 1 0.5 cc. toxin	Dec. 1			
		2 hours later...	4,830,000	58	Many microcytes
		6 hours later...	3,820,000	56	
		Dec. 2.....	3,710,000	50	Many microcytes (fig. 1)
		Dec. 3.....	3,790,000	50	
		Dec. 8.....	3,390,000	52	Many macrocytes and poikilocytes (fig. 1)
		Dec. 11.....	3,680,000	60	
		Dec. 17.....	4,600,000	64	Mostly normal (fig. 1)
	Immunizing dose 2 0.5 cc. toxin	Dec. 18			
		6 hours later...	3,580,000	50	Many microcytes
		Dec. 19.....	3,170,000	50	
		Dec. 20.....	3,820,000	53	Some macrocytes and poikilocytes
		Jan. 6.....	5,560,000	71	Normal
	Immunizing dose 3 0.5 cc. toxin	Jan. 6			
		6 hours later...	5,590,000	78	Normal
		Jan. 7.....	5,770,000	78	Normal
	Immunity testing dose 0.5 cc. toxin	May 30 (4½ months after dose 3)			
		6 hours later...	5,310,000	58	Many microcytes (fig. 2)
		May 31.....	5,570,000	44	
		June 1.....	4,860,000	54	
		June 3.....	4,260,000	51	Few macrocytes (fig. 2)
		June 13.....	4,060,000	79	Few macrocytes (fig. 2)

The Establishment of Immunity.—The result of the initial intravenous inoculation with toxin was similar in each of the animals. At the end of the second hour after the inoculation there was a decided decrease in the number of red cells, a drop in the hemoglobin per-

centage accompanied by an alteration in the red cell structure consisting in the appearance of many small microcytes and often a number of poikilocytes. These symptoms became more marked during the first 48 hours following the inoculation. As the red cell count began again to increase, the microcytes were gradually replaced by large macrocytes accompanied in most cases by a certain amount of polychromatophilia. Following the inoculations the red cell count became normal in from two to four weeks. As the cell count returned to normal, the structure of the cells also returned to the normal. Further details of these changes are indicated in the table and in figure 1, and they were discussed at some length in former papers.²

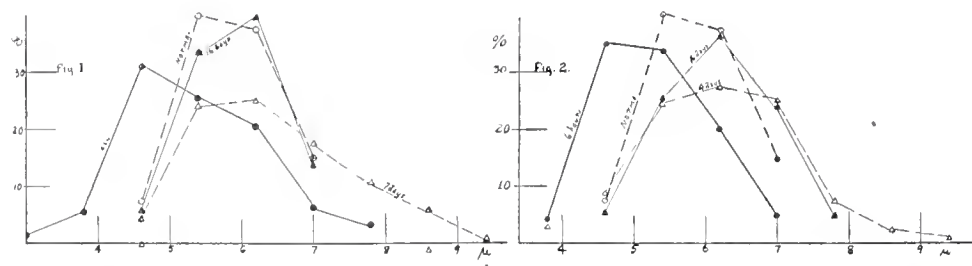
As soon as the animal showed a normal red cell count and normal red cell structure, the initial inoculation was repeated. The results in most cases were similar to those following the first inoculation as the four cases quoted indicate, except that the drop in red cell count and hemoglobin was not so marked and the modification in the form of the red cells was less conspicuous. The recovery was slightly more rapid than following the first inoculation. In three cases the animals did not react to the second inoculation of toxin and were therefore considered to have developed an immunity. The ten animals which reacted to the second inoculation were given a third under exactly the same conditions as the second. In all ten cases, the third inoculation failed to produce any reaction. In these animals two inoculations evidently provided an immunity.

The Duration of Immunity.—To determine the duration of the immunity thus established subsequent inoculations were made at intervals of one to seven months. In each case the same sample of toxin as used in the initial inoculation of an animal was used in this test reinoculation. As previously noted all the toxins were stored in completely filled and sealed bottles and aged for some weeks before they were initially used in the immunization. All of the samples used in the experiments remained unchanged from the first test to the time they were used for reinoculation. Comparatively fresh toxin and toxin that had stood in sealed bottles for 4 to 6 months produced the same reaction in rabbits as determined by the decrease in red cells, hemoglobin percentage, and by the degree of anocytosis. The in vitro hemolytic titer also remained unchanged. This is in conformity with Neill's⁴ results with the keeping quality of *B. welchii* toxin in filled and sealed containers.

⁴ Neill, J. M.: J. Exper. Med., 1926, 44, p. 215.

The first reinoculation was made seven weeks after the establishment of immunity, rabbit 1 (table). The same dosage of the same toxin which initially produced a marked reaction, seven weeks after the establishment of immunity failed to produce any change in the red cell count, hemoglobin or red cell structure. The immunity must therefore have persisted in this animal for seven weeks.

A group of five immunized rabbits were reinoculated with the same toxin as used in the immunization, $4\frac{1}{2}$ months after the last primary inoculation. In one of the five (rabbit 2 in table), the reinoculation produced no decrease in red cell count in hemoglobin percentage and no definite change in red cell structure as shown in more detail in the case history. It was therefore, assumed that the immunity of this animal had remained without impairment for the $4\frac{1}{2}$ months.



Variations in red cells of rabbit 4 after initial intravenous injection of *B. welchii* toxin (fig. 1), and after a similar injection $4\frac{1}{2}$ months after establishing immunity (fig. 2), respectively. Ordinates represent the percentage occurrence of each size group; and abscissas the diameters in microns. In figure 1, --0-- is for the rabbit before injection; --●-- 1 day after injection; --△-- 7 days after injection; and --△-- 16 days after injection. In figure 2, --0-- is for the immune rabbit before reinoculation; --●-- for 6 hours after reinoculation; --△-- four days after reinoculation; and --△-- 16 days after reinoculation.

Four of the five rabbits in this group however, showed a definite reaction to the reinoculation. The reactions of 2 rabbits (3 and 4 in table) were characteristic. The decrease in the red cell count following the reinoculation though less than following the initial inoculations are nevertheless conspicuous, in contrast with the absence of reaction to the third immunizing dose. The change in the structure of the red cells following the reinoculation of these animals was more definite and approximated the degree of anisocytosis following the primary inoculation.

Figure 1 indicates the variation in red cell size in the normal rabbit (3 of table) and at intervals following the first inoculation with toxin. These curves emphasize the statement previously made that the first effect of the toxin is the production of many microcytes followed by

increase in macrocytes. Figure 2 indicates the reaction of the same animal to the toxin $4\frac{1}{2}$ months after the last immunizing dose of toxin. It is apparent from the curves that the anocytosis produced at this interval after immunization is almost as great as following the initial dose of toxin. Similar curves have been obtained from a study of the blood of several animals following the primary and the reinoculations. These also show that although the decrease in red cell count was less marked than following the initial inoculation the anocytosis is almost as great as in the normal animal, thus it may be concluded, that the immunity in these animals had very considerably decreased after a lapse of four and one half months.

Two animals were reinoculated 6 months, and one rabbit 7 months, after the last immunizing dose of toxin. All of these animals showed some decrease in red cell count, although less than following the initial inoculation and a decided anisocytosis though also less than following the first dose of toxin. Since they had all failed to react to the last immunizing dose there must have been a partial loss in immunity during this time.

In all, 9 immunized rabbits were reinoculated at various periods after the establishment of immunity. Two of the nine rabbits exhibited no decrease in immunity, one 7 weeks, and one $4\frac{1}{2}$ months, after immunization. The remaining 7 animals all showed a conspicuous decrease in the immunity after $4\frac{1}{2}$ to 7 months. This is a very small group of animals but the results are apparently sufficiently definite to warrant the conclusion that the duration of *B. welchii* antitoxic immunity in rabbits is comparatively short. If *B. welchii* is concerned in pernicious anemia in man the remission in the disease might be accounted for as transient periods of antitoxin immunity.

SUMMARY

It has been shown that the injection of *B. welchii* toxin into rabbits in doses sufficient to produce a definite anemia is followed, after one to two doses by the establishment of an immunity. The immunity remains unimpaired for more than seven weeks but shows a conspicuous decrease after 4 to 7 months.

ACTION OF HEMOTOXINS ON OXYGENATED AND REDUCED BLOOD

1. *B. welchii* TOXIN

GUILFORD B. REED, J. H. ORR AND W. A. CAMPBELL

From the Department of Bacteriology, Queen's University, Kingston, Ontario, Canada

It was recently shown by Neill¹ that the exposure of certain bacterial culture filtrates containing hemotoxins to the air results in an oxidation which renders the toxins inactive and that the oxidized filtrates may be reactivated by reduction with reducing agents as sodium hyposulphite or through the anaerobic action of bacteria. We have repeated certain of these procedures with results which are in general in conformity with those of Neill. As the test for hemotoxin consists in bringing the toxin into contact with red cells it seemed probable that a consideration of the capacity of the hemoglobin to absorb or give up oxygen might yield significant results. Accordingly we have tested the action of hemotoxins, which had been protected from the air and which had been oxidized, on oxygenated and reduced blood. The present paper deals with the action of *B. welchii* toxin.

Procedure.—Rabbit blood was defibrinated by shaking with glass beads, the red cells washed three times in Ringer's solution and diluted in Ringer's solution to make a 5% suspension. For reduced blood cells this suspension was treated in one of three ways: carbon dioxide was bubbled through the suspension for 20 minutes; the suspension was held in an anaerobic jar for 48 hours; or the washed red cells were diluted to a 5% emulsion in equal parts of Ringer's solution and sterile beef infusion broth and held for 48 hours and under a vaseline seal. The blood cell suspension in each case developed the characteristic color of reduced hemoglobin. Where the reduced cell suspensions were to be compared with oxygenated blood the freshly prepared suspensions were divided into two portions one preserved in a shallow layer in Erlenmeyer flasks loosely stoppered with cotton and the other portion subjected to reduction. The oxygenated and the reduced samples were then tested at the same time.

The *B. welchii* toxins consisted of filtrates of anaerobic cultures in a chopped meat medium prepared according to the method of Bengston. The filtration was always rapid and the filtrate was immediately dispensed into small bottles with the least possible exposure to air and covered with a vaseline seal. The toxins used in these experiments were the same as those used in our work on *in vitro* and *in vivo* anemia.² Samples of toxins were oxidized where required

Received for publication, Sept. 1, 1927.

¹ J. Exper. Med., 1926, 44, pp. 119, 215.

² Reed, G. B.; Orr, J. H., and Spence, M.: J. Infect. Dis., 1927, 41, p. 283; Reed, G. B., and Orr, J. H.: Ibid., p. 289.

according to Neill's procedure by exposing them to the air in Erlenmeyer flasks loosely stoppered with cotton for 24 hours immediately before use.

All the hemolytic tests were made in 1 cc. amounts of fluid. The toxins which had been preserved under vaseline were diluted in recently boiled Ringer's solution, the oxidized toxin in aerated Ringer's. The dilutions were sufficient to give final concentrations in the test tubes of 1:20 to 1:400, 0.9 cc. were added to each tube and 0.1 cc. of the suspension of red cells. Where reduced blood was used the tubes were sealed with vaseline as soon as the blood was added. The tubes were incubated in a water thermostat at 37 C. for five hours and the results read after standing over night in the ice box or after centrifuging. In the accompanying tables where 20% or more of the cells were hemolyzed the result is recorded as ++ where there was definite hemolysis but less than this amount it is recorded as + and the absence of hemolysis as 0.

Reduced and Oxidized Toxins and Oxygenated Blood.—A sample of *B. welchii* toxin (1) freshly prepared and preserved under a

TABLE 1
HEMOLYSIS OF OXYGENATED RED BLOOD CELLS BY OXIDIZED AND REDUCED *B. WELCHII* TOXIN

	Toxin Dilutions						Control (Without Toxin)
	1:20	1:40	1:60	1:80	1:100	1:200	
Toxin 1							
Oxidized.....	++	+	0	0	0	0	0
Reduced.....	++	++	++	+	0	0	0
Toxin 2							
Oxidized.....	++	++	+	0	0	0	0
Reduced.....	++	++	++	++	+	0	0
Oxidized and again reduced by Na ₂ S ₂ O ₃	++	++	++	++	+	0	0

++ = definite hemolysis; + = somewhat less hemolysis, and 0 = absence of hemolysis in all tables.

vaseline seal was exposed to the air in a shallow layer in an Erlenmeyer flask for 24 hours. The oxidized sample was then compared with a sample of the same filtrate, which had remained sealed, on freshly washed, oxygenated red cells. The hemolytic titer of each is shown in table 1, together with results with a second sample (2), treated in the same way. It is apparent from the figures in the table that exposure of these filtrates to the air for 24 hours results in a reduction of the hemolytic activity to approximately half the value exhibited by the unexposed toxin. Qualitatively this corresponds with the results obtained by Neill though he reports a relatively greater degree of destruction of toxin by oxidation. Neill suggests that the oxidation may not be direct but results from the autoxidation of some other substance in the filtrate which in turn acts as the toxin oxidizing agent. It is possible that our filtrates contain less of the autoxidizable sub-

stance. Some ingredients may inhibit the oxidation. Neill found a P_H lower than 7.5 to retard the reaction. Toxin 1 used in the previous experiment had a P_H of 7.4, and toxin 2 had a P_H of 7.2.

Treating toxin 2 after oxidation for 20 minutes with a freshly prepared 0.1% solution of $\text{Na}_2\text{S}_2\text{O}_3$ resulted in a return to its original hemolytic activity.

Reduced and Oxidized Toxins in the Hemolysis of Reduced and Oxygenated Blood.—In carrying out the experiments recorded in the preceding section it occurred to us that if the reduced toxins or those which have been protected from the air are so readily oxidized on exposure to the air, contact with fully oxygenated red blood cells might result in a corresponding oxidation, or conversely that contact of

TABLE 2
HEMOLYSIS OF OXYGENATED AND REDUCED RED BLOOD CELLS BY OXIDIZED AND REDUCED B. WEICHHI TOXIN

	Toxin Dilutions						Control (Without Toxin)
	1:20 and 1:40	1:60	1:80	1:100	1:200	1:400	
Toxin 2							
Oxidized, plus oxygenated blood...	++	+	0	0	0	0	0
Oxidized, plus reduced blood.....	++	++	++	+	0	0	0
Reduced, plus oxygenated blood...	++	++	+	0	0	0	0
Reduced, plus reduced blood.....	++	++	++	++	++	0	0
Toxin 1							
Oxidized, plus oxygenated blood...	++	++	0	0	0	0	0
Oxidized, plus reduced blood.....	++	++	++	++	++	+	0
Reduced, plus oxygenated blood...	++	++	+	0	0	0	0
Reduced, plus reduced blood.....	++	++	++	++	++	+	0

oxidized toxin with reduced blood might result in reduction of the toxin. To test this suggestion samples of the toxins previously used were brought into contact with fully oxygenated red cells and with portions of the same suspensions of red cells which had been reduced until they assumed the characteristic dark purple color. Results are indicated in table 2. From the data of the table it is apparent that a very much higher concentration of oxidized toxin is required to hemolyze oxygenated blood than reduced blood. Reduced toxin as in the former case proved more effective than the oxidized toxin but at the same time a much higher concentration of the reduced toxin is required to hemolyze the oxygenated blood cells than the reduced cells. Though the examples quoted in table 2 both show that the reduced blood is hemolyzed by a much lower concentration of the toxin than is required by the oxygenated blood the proportionate amount of toxins for the reduced and oxygenated blood is very different in the two examples. This has been repeatedly observed and may result from

differences in the degree of reduction of the blood cells, as the data of table 3 suggest, or from a difference in the oxidizibility of different filtrates.

These results permit of at least two interpretations. The red cells through the activity of the hemoglobin may oxidize or reduce the toxin according to the state of oxygenation of the hemoglobin. On the other hand the reduced blood, perhaps the reduced hemoglobin, may more

TABLE 3

COMPARISON OF THE HEMOLYSIS WITH WELCHII TOXIN 2 OF REDUCED BLOOD CELLS, WITH FRESH OXYGENATED AND REOXYGENATED BLOOD

Blood Cells	Toxin Dilutions						Control (Without Toxin)
	1:20 and 1:40	1:60	1:80	1:100	1:200	1:400	
Fresh oxygenated.....	++	+	0	0	0	0	0
Reduced in anaerobic jar.....	++	++	++	++	+	0	0
Reoxygenated.....	++	++	++	+	0	0	0
Reduced in CO ₂	++	++	++	++	++	+	0
Reoxygenated.....	++	++	++	+	0	0	0
Reduced in broth.....	++	++	++	++	+	0	0
Reoxygenated.....	++	++	+	0	0	0	0

TABLE 4

HEMOLYSIS OF REDUCED AND OXYGENATED BLOOD IN HYPOTONIC SALT SOLUTION

Blood	% Concentration NaCl		
	0.50 and 0.55	0.60	0.65 to 0.85
Fresh oxygenated.....	++	±	0
Reduced, CO ₂	++	±	0
Reoxygenated.....	++	±	0

readily absorb toxin than the oxygenated blood or hemoglobin. This problem will be considered in a later paper.

Reduced Toxins in the Hemolysis of Blood Reduced by Different Methods.—Samples of the same suspension of washed red cells were subjected to reduction by CO₂, by exposure in an anaerobic jar and by exposure under a vaseline seal as described earlier. The treatment in each case was continued until the three lots assumed a similar dark purple color. Portions of each reduced sample were then exposed to the air in a shallow dish until the color of oxygenated blood was regained. The concentration of reduced toxin 2 necessary to produce hemolysis of the fresh oxygenated, the reduced and the reoxygenated blood was then determined, with the results shown in table 3.

It is apparent that the different methods of reduction yield similar but somewhat divergent results probably due to different degrees of reduction. Similar results have been observed with other samples of toxins.

Hemolysis of Reduced and Oxygenated Blood by Hypotonic Salt Solution and by Ammonia.—In order to demonstrate whether or not the processes of reduction had any influence on the fragility of the red cells portions of the same suspensions of washed cells which have been kept freely exposed to the air, reduced to a deep purple color, and reduced and reoxygenated have respectively been tested in a series of concentration of salt. Table 4 records the result of a characteristic experiment. Salt solutions of the concentrations noted in the table were added to a series of tubes, 0.9 cc. per tube and 0.1 cc. of blood cell suspension added, no correction for the salt concentration of the cell suspension was made as the error was the same in the three preparations. It is apparent from such experiments that reduction and reoxygenation have not rendered the cells more easily hemolyzed in hypotonic solutions. Similar tests with varying concentrations of ammonia have clearly indicated that reduction has not rendered the cells more easily hemolyzed by this reagent.

SUMMARY

In agreement with earlier work of Neill it was found that the oxidation of *B. welchii* toxin by exposure of toxic filtrates to the air for 24 hours results in considerable loss of hemotoxic action.

The hemolysis of oxygenated red cell suspensions requires a much higher concentration of *B. welchii* toxin than reduced red cell emulsions. It is suggested that this may result from an oxidation—reduction relationship between the toxin and hemoglobin or that it may depend upon a difference in the absorption of toxin by oxygenated and reduced blood.

AN EPIZOOTIC OF HEMORRHAGIC SEPTICEMIA IN GUINEA-PIGS

GUILFORD B. REED AND G. H. ETTINGER

From the Department of Bacteriology, Queen's University, Kingston, Ontario, Canada

An epizootic recently broke out in one large room of our animal house involving a colony of 134 guinea-pigs. Most of these guinea-pigs had been reared in this room and all had been kept under the same conditions for several months except a few males which were acquired from a commercial breeder a few weeks before the beginning of the epizootic. The breeder reports no disease among his animals but this has not been verified.

The epizootic appeared abruptly and disappeared gradually as shown in the following tabulation, in terms of the number of deaths per day.

	November								December							
Date	23	24	25	26	27	28	29	30	1	2	3	4	5	6	7 to Jan. 1	
Number of deaths.....	8	11	15	12	10	8	3	0	2	6	0	0	2	3	0	(total 80)

There may have been a few deaths from the disease before the first day noted in the table. Approximately 60% of the guinea-pigs in the colony died during a period of two weeks. Those which survived the two-weeks epizootic have shown no symptoms of a recurrence of the disease during the subsequent eight months. Other guinea-pigs in the same building but in separate rooms did not contract the disease. About 25 rabbits and 50 white mice in the room with the diseased guinea-pigs for several months previous to and during the epizootic showed no symptoms of having contracted the infection.

The symptoms of the disease consisted in lassitude and a slight fever lasting for one to two days preceding death. At necropsy the animals showed acute swelling of the lymph nodes and spleen, hyperemia of the serous membranes and intestinal mucosa with areas of hemorrhage and extensive hemorrhagic edema of the lungs. The lungs microscopically showed intense injection, some hemorrhage into the alveoli and small interstitial bronchopneumonic patches with no exudate. Animals which

had been exposed but which showed no physical signs of the disease were killed and their lungs examined. There was less capillary injection, but considerable interstitial bronchopneumonia with some slight exudate in the bronchioles and a tendency to fibrosis. This is probably a late manifestation of the disease, not in itself fatal, and may be an aggravation of a chronic condition, since guinea-pigs not exposed to the disease frequently show small bronchopneumonic patches.

An organism of the hemorrhagic septicemia group was isolated from several of the animals dead or ill of the epizootic disease and used to reproduce the disease in guinea-pigs from an uninfected colony. The organism was isolated on two occasions from the heart blood of moribund animals, other animals in an apparently similar condition gave negative blood cultures but the hemorrhagic areas of the lungs or serous membranes of all the animals examined gave positive cultures. The primary growth on fresh blood agar was rather slow at 37 C. only appearing after 36 to 48 hours. In subsequent generations the growth was much more rapid. On plain agar the colonies are circular, 1 to 2 mm. in diameter, slightly raised, moderately opaque and gray white, carbohydrates are not fermented, milk is unchanged, no indol is formed. Growth on potato is similar to that on agar. On blood agar in 24 to 48 hours there is an extensive zone of hemolysis about the colonies. On all the mediums the organisms were quite irregular in form and size ranging from spherical forms about 0.2 to 0.6 μ , frequently in diploids, to rods up to 1.5 to 2 μ many of the rods are club or wedge shaped and when stained with methylene blue most of the rods show irregular polar granules. Gram's method always failed to stain. When smears are made from the body lesions or from cultures on media containing serum the organisms show delicate capsules.

The intraperitoneal injection of young broth cultures, two to three generations from the original isolation, in 0.2 to 0.3 cc. amounts in mature guinea-pigs from an uninfected colony caused death in 18 to 24 hours, with symptoms similar to those described above. Similar organisms were isolated from the inoculated animals after death. Intratracheal inoculation resulted in death in 4 to 7 days with similar symptoms. After 10 to 12 transfers on agar slants during a period of eight months the organism completely lost its virulence for guinea-pigs.

The organism closely resembles *Pasteurella cuniculida* but produces a somewhat heavier growth on solid mediums and is not to any extent pathogenic for rabbits.

SUMMARY

An epizootic is described involving the death of 60% of a colony of guinea-pigs. An organism resembling *Pasteurella cuniculida* was isolated from animals in the epidemic, which when injected into unexposed guinea-pigs reproduced the disease.

EFFECT OF CL. SPOROGENES ON TOXIN PRODUCTION BY CL. BOTULINUM

E. WAGNER SOMMER AND KATHRYN GLUNZ

*From the George Williams Hooper Foundation for Medical Research, University of California
Medical School, San Francisco*

Experimental results and opinions do not agree as to the effect of other organisms on the growth and toxin production of *Cl. botulinum*. Dubovsky and Meyer¹ observed in the course of their experimental work on the isolation of *Cl. botulinum* "that a few viable spores accidentally introduced as contaminations may produce toxic cultures" and that "repeated observations have shown that the poison may develop in mixtures, notwithstanding the presence of other aerobic or anaerobic bacteria." Hall and Peterson² noted that "aerobic bacteria found in certain soil samples were able to prevent the accumulation of strong toxin in the cultures," and that acid-producing aerobes inhibited toxin production by *Cl. botulinum* in glucose broth, although no such inhibition occurred in plain broth.

B. sporogenes is admittedly one of the most common contaminants of *Cl. botulinum* cultures. Reddish³ for example, found that 18 of 19 stock strains contained *sporogenes* and attributes the development of nontoxic cultures to the proportion in which the contaminant is present. Gunnison and Schoenholz⁴ have shown, however, by complement fixation tests that nontoxic *Cl. botulinum* cultures retain their serologic characteristics, and therefore need not be considered contaminated cultures.

Jordan and Dack⁵ inoculating simultaneously known numbers of *sporogenes* spores with *Cl. botulinum* spores into meat medium conclude that "*Cl. sporogenes* may either prevent the development of botulinum toxin altogether, may diminish the amount that is produced or may cause the early disappearance of the toxin."

The effect of *sporogenes* on botulinum grown in vegetable as well as in meat medium has been studied in this laboratory. The results sub-

Received for publication, Aug. 22, 1927.

¹ J. Infect. Dis., 1922, 31, p. 501.

² J. Bact., 1923, 8, p. 319.

³ J. Infect. Dis., 1921, 29, p. 120.

⁴ J. Immunol., 1927, 13, p. 79.

⁵ J. Infect. Dis., 1924, 35, p. 576.

stantiate the findings of Jordan and Dack in so far as sporogenes does weaken the toxin titer, the poison may be formed, however, wherever *Cl. botulinum* is present.

Experimental.—Spore suspensions of *Cl. botulinum* (strain 97 isolated in 1921 from home canned peas) and of *Cl. sporogenes* (strain 46 described by I. C. Hall⁶) were used in all of the experiments. The organisms were grown in peptic digest broth with 2% Bena peptone. This medium consistently yielded good spores (50-80% of the total number of organisms). After 10 days' incubation at 37 C., the cultures were stored at room temperature. The supernatant fluid was decanted; the spores were removed from the medium by centrifugation, and suspended in a sterile M/15 phosphate solution of P_H 7.0. Clumps were broken up by shaking with sterile sand.

The suspensions were standardized by the Breed⁷ method of counting, employing Burke's⁸ staining technic. Immediately before inoculation the spores were heated in the phosphate solution for one hour at 80 C., to detoxify the suspension and kill the vegetative forms.

Two types of mediums, beef heart (Robertson's⁹ "cooked meat medium") and spinach or asparagus vegetable infusion, were used. The latter were prepared as follows: the raw fresh vegetable was ground and mixed with tap water in the proportion of two liters to each kilogram of material. This was brought to a boil slowly, the reaction adjusted to P_H 7.6 with sodium hydroxide and carefully stabilized. It was tubed with considerable of the solid vegetable particles, stratified with vaseline, and sterilized in the autoclave at 15 pounds for 30 minutes.

Duplicate series of each of the three mediums, beef heart, spinach, and asparagus, were inoculated simultaneously with varying numbers of heated botulinum and sporogenes spores as shown in tables 1 and 2. The procedure is essentially the same as that used by Jordan and Dack.

The mediums with a few exceptional tubes supported vigorous growth. After 10 days' incubation samples were removed and centrifugated for toxin determinations. Guinea-pigs were used to detect the presence of toxin without any attempt to determine the exact potency. The intraperitoneal injections were regulated according to the weight of the animal using 1 cc. of the undiluted culture fluid for a 250 gm. guinea-pig. The approximate strength of the toxin was determined on white mice (table 2), 1 cc. of different dilutions being the dose for a 20 gm. mouse injected intraperitoneally.

Results.—From tables 1 and 2 it will be noted that toxin was produced in every tube of the meat cultures where *Cl. botulinum* was present. Spinach yielded a toxin nearly as strong as that in the meat, even though the macroscopic evidence of growth was slight in a few instances. Irregularity of growth in vegetable mediums has been observed by other workers.¹⁰ Asparagus did not favor the formation of toxin so readily. (It should be emphasized that there was evidence of active growth with the production of considerable gas within 48 hours in all of the asparagus tubes.)

⁶ J. Infect. Dis., 1922, 30, p. 445.

⁷ Am. Pub. Health Assn. Standard Methods of Milk Analysis, 1923.

⁸ J. Infect. Dis., 1923, 32, p. 433.

⁹ J. Path. & Bact., 1916, 20, p. 327.

¹⁰ Schoenholz, P.; Esty, J. R., and Meyer, K. F.: J. Infect. Dis., 1923, 33, p. 289. Koser, S. A.; Edmondson, R. B., and Giltner, D. V. N.: J. Am. M. A., 1921, 77, p. 1250. Dickson, E. C.: Monograph 8, Rockefeller Inst. for M. Res., 1918. Geiger, J. C.; Dickson, E. C. and Meyer, K. F.: Pub. Health Bull. 127, 1922.

Table 2 shows the toxin titers of the three mediums, as determined on mice. Certain tubes, 1, 8, 15, 22, 29, and 36-43 of table 1 were chosen for these tests as representative. The apparent discrepancy of the toxin tests determined on mice and guinea-pigs is explained by the fact that mice per gram weight are twice as resistant to the poison (Bengston¹¹). One cc. of a 1:10 dilution was

TABLE 1
TESTS FOR BOTULINUM TOXIN PRODUCED IN ASPARAGUS, SPINACH, AND MEAT MEDIUMS

Tube	Number of Spores Inoculated		Effect on Guinea-pigs		
	Botulinum	Sporogenes	Asparagus	Spinach, Hours	Meat, Hours
1	0	10,000,000	0	0	0
2	100	0	0	+ <16	+ 5-6
3	100	100	0	±	+ 6
4	100	1,000	0	±	+ 6-13
5	100	10,000	0	+ 18	+ 6-13
6	100	100,000	0	±	+ 6-13
7	100	1,000,000	0	±	+ 15
8	100	10,000,000	0	±	+ 6-13
9	1,000	0	+	+ 16	+ 5-6
10	1,000	100	±	+ 18	+ 14
11	1,000	1,000	0	+ 24	+ <14
12	1,000	10,000	0	+ 24	+ <14
13	1,000	100,000	0	+ 19	+ <30
14	1,000	1,000,000	0	+ 40	+ 18-24
15	1,000	10,000,000	0	+ 40	+ <14
16	10,000	0	+	+ 16	+ 3½
17	10,000	100	±	+ 22	+ <43
18	10,000	1,000	0	+ 19	+ <14
19	10,000	10,000	0	+ 24	+ <14
20	10,000	100,000	0	±	+ 15
21	10,000	1,000,000	0	±	+ 17
22	10,000	10,000,000	0	±	+ 24
23	100,000	0	+	+ <15	+ 6
24	100,000	100	+	+ 19	+ 5
25	100,000	1,000	0	+ 36	+ <14
26	100,000	10,000	0	+ 48	+ <14
27	100,000	100,000	0	+ 36	+ <14
28	100,000	1,000,000	0	+ 24	+ <18
29	100,000	10,000,000	0	±	+ 30
30	1,000,000	0	+	+ 5	+ <9
31	1,000,000	100	+	+ <18	+ <9
32	1,000,000	1,000	0	+ 18	+ <9
33	1,000,000	10,000	0	+ 24	+ <9
34	1,000,000	100,000	0	+ 24	+ <24
35	1,000,000	1,000,000	0	±	+ 12
36	1,000,000	10,000,000	0	±	+ 15
37	10,000,000	0	+	+ <12	+ <18
38	10,000,000	100	+	+ 18	+ <18
39	10,000,000	1,000	+	+ <16	+ <18
40	10,000,000	10,000	+	+ <16	+ <18
41	10,000,000	100,000	0	+ 24	+ <18
42	10,000,000	1,000,000	0	+ <16	+ 33
43	10,000,000	10,000,000	0	±	+ <18

+ indicates death of guinea-pig; 0, survival; and ±, symptoms but survival, in tables 1 and 2.

the highest toxin dose selected in order to avoid any injurious effect of other metabolic products in the culture fluids. The results of the tests on guinea-pigs for these 13 cultures in each medium are included in this table for purposes of comparison. The tabulated data are only part of a larger series of experiments with analogous results and are chosen as typical.

¹¹ U. S. P. H. S. Hyg. Lab. Bull. 136, 1924, p. 70.

DISCUSSION

Jordan and Dack found that "when *Cl. sporogenes* is considerably in excess in beef heart mediums no botulinus toxin is formed." This fact is not entirely substantiated by the results herein reported as in every meat tube *Cl. botulinum* liberated sufficient toxin to kill a guinea-pig irrespective of the number of *sporogenes* spores present. The discrepancy might be attributed to the respective strains employed in the two laboratories since it is well known that different strains of *Cl. botulinum* exhibit varying serologic and biochemical characteristics; furthermore, some may be more easily affected by the presence of other

TABLE 2
TESTS ON MICE AND GUINEA-PIGS FOR TOXIN PRODUCED IN THE THREE MEDIUMS

Tube (See table 1)	Number of Spores Inoculated		Asparagus		Spinach		Meat	
	Botulinum	Sporogenes	Mice Dilution of Toxin	Guinea-pigs Undiluted Toxin	Mice Dilution of Toxin	Guinea-pigs Undiluted Toxin	Mice Dilution of Toxin	Guinea-pigs Undiluted Toxin
1	0	10,000,000	1:10	0	1:10	0	1:10	0
8	100	10,000,000	1:10	0	1:10	0	(1:100 \pm)	0
15	1,000	10,000,000	1:10	0	1:10	\pm	1:10	\pm
22	10,000	10,000,000	1:10	0	1:10	\pm	1:10	\pm
29	100,000	10,000,000	1:10	0	1:10	0	1:10	\pm
36	1,000,000	10,000,000	1:10	0	1:10	0	1:10	\pm
43	10,000,000	10,000,000	1:10	0	1:10	+	1:10	+
42	10,000,000	1,000,000	1:10	0	1:500	+	1:10	+
41	10,000,000	100,000	1:10	0	1:100	+	1:10	+
40	10,000,000	10,000	1:100	+	1:100	+	1:10,000+	+
39	10,000,000	1,000	1:100	+	1:100	+	1:10,000+	+
38	10,000,000	100	1:100	+	(1:500 \pm)	+	1:1,000+	+
37	10,000,000	0	1:100	+	1:1,000+	+	(1:10,000 \pm)	+
					(1:5,000 \pm)		1:10,000+	
							(1:50,000 \pm)	

organisms or may lose their toxicity spontaneously which occurs not infrequently with B and C type strains. Strains of *Cl. botulinum* as well as *Cl. sporogenes* vary widely in their heat resistance so that in one case the botulinum spores may be more resistant than the sporogenes and the reverse might hold true in another experiment and thus account for the disparity of results. It is unfortunate that no viable counts were made to check the microscopic counts.

The inoculation of an equal or greater number *Cl. botulinum* with *Cl. sporogenes* in spinach medium produced toxin with but one exception, when only a hundred spores of each were used. In a few cases (table 1: tubes 14 and 15) the introduction of an overwhelming number of sporogenes spores did not prevent the development of toxin. The deleterious influence of sporogenes is most marked in the asparagus

medium. This is not surprising since *Cl. botulinum* produced a considerably weaker toxin in this pabulum than in the others tested. In all three mediums the toxin strength is gradually diminished by increasing the number of sporogenes. These results are amply verified in experiments which are not detailed in this paper but may be indicated as follows:

	RELATIVE NUMBER OF ORGANISMS	
	Toxicity for Mice in Dilution of	
	Meat Medium	Spinach Medium
Botulinum	1:50,000	1:50,000
Botulinum : Sporogenes = 1:1	1:500	1:1,000
Botulinum : Sporogenes = 1:2	1:100	Not tested
Botulinum : Sporogenes = 1:100.....	Not tested	1:100

Attempts were made to determine whether one organism had a tendency to outgrow the other. Shake tubes and pour plates proved unsatisfactory for differential colony counts so surface plates were made. Several types of colonies developed but clear cut differences could not be recognized. This presents a problem for further studies.

The metabolic products of *Cl. sporogenes* are probably not injurious to botulinus toxin since the supernatant fluid of a culture of the non-toxic anaerobe, when added to botulinus toxin, did not destroy it, for mice died in a few hours after being injected.* Dack¹⁵ likewise made a similar observation and states, "it may be seen that the sporogenes filtrate has little if any effect on the potency of the botulinum toxin even after 10 days incubation." Various attempts to find a selective medium for botulinum have shown that sporogenes exhausts a substrate of the nutritive substance required for a subsequent vigorous growth of *Cl. botulinum*. Planted together it is not unlikely that they contend for the same food substance, some of which may be necessary for toxin production, and if botulinum fails in securing it little if any toxin is formed. It is barely possible that longer incubation may give different results.

SUMMARY

Spore suspensions of *Cl. botulinum* and *Cl. sporogenes* inoculated in varying relative amounts into meat, spinach and asparagus mediums which are incubated for 10 days and tested for the presence of toxin,

* One cc. of the undiluted mixture of sporogenes and botulinum supernatants from asparagus and spinach mediums was injected, and a 1:10 dilution of the mixture from beef heart medium.

¹⁵ J. Infect. Dis., 1926, 38, p. 165.

reveal botulinum toxin in all the meat cultures in which *Cl. botulinum* is present irrespective of the number of sporogenes. The inoculation of an equal or greater number of *Cl. botulinum* with sporogenes is usually necessary for toxin production in spinach. In asparagus, a poorer toxin medium, a more deleterious influence of sporogenes on botulinum toxin has been noted. In meat, spinach and asparagus mediums increasing numbers of sporogenes gradually diminish the strength of the toxin.

THE PRODUCTION OF LOCAL RENAL LESIONS IN RABBITS BY INTRAVENOUS INJECTIONS OF CERTAIN STRAINS OF B. COLI

ONE PLATE

HENRY F. HELMHOLZ

From the Section on Pediatrics, Mayo Clinic, Rochester, Minnesota

Since 1915 I have been interested in studying infections of the urinary tract in children. In attempting to produce urinary infection in rabbits we used a large number of organisms isolated from the urine of patients with pyelitis. In only about 12% of the animals that received injections of colon bacilli was it possible to demonstrate local lesions in the kidney. Injection of a combination of colon bacilli and an organism of the streptococcus group somewhat increased the number of positive results. On the whole, however, the results were negative for we were looking for a constant method of producing infection of the urinary tract so that the curative value of drugs recommended for treatment of pyelitis could be studied. We were studying the effect of chilling rabbits after intravenous injection of organisms when, in testing the animals before injection, we found a female whose urine contained large numbers of pus cells and innumerable organisms of the colon bacillus group.

Spontaneous nephritis in the rabbit is well known and a certain number of cases will be found in any large group of animals examined. Spontaneous pyelitis in the rabbit has not been described, and little has been written on bacilluria in rabbits. The pathologic and histologic changes produced by the intravenous, as well as intracystic, injection of colon bacilli isolated from the urine of the rabbit mentioned, have been reported elsewhere. In this paper I wish to emphasize the specific affinity for rabbit kidney shown by some strains of colon bacilli when injected intravenously.

The work of Rosenow,¹ Haden,² Bargaen,³ Nickel,⁴ Meisser and Bumpus,⁵ and others, has shown that streptococci, isolated from foci

Received for publication, Sept. 10, 1927.

¹ J. Am. M. A., 1914, 63, p. 1835.

² Am. J. M. Sc., 1926, 172, p. 885.

³ J. Am. M. A., 1924, 83, p. 332.

⁴ Ibid., 1926, 87, p. 1117.

⁵ Arch. Int. Med., 1921, 27, p. 326.

in patients suffering from certain diseases, tend to produce the same disease in animals when injected intravenously. Arthritis, myositis, endocarditis, pyelonephritis, chronic ulcerative colitis, and other diseases have been experimentally reproduced in animals. Up to this time no one has shown that particular strains of colon bacilli injected intravenously in rabbits have a particular tendency to produce local lesions in any specific organ or tissue.

Exper. 1.—Into 66 animals were injected eight different strains of colon bacilli isolated from the urine of patients with pyelitis. A variety of lesions was produced in different organs. In 26 animals there was evidence of focal involvement. The kidney was involved in eight instances, the cecum in seven, the gallbladder in seven, the appendix in five, and the stomach in four. There were also single instances of hemorrhagic infarction of the colon, ulcerative colitis, hemorrhagic enteritis, and duodenal hemorrhage. Although the kidney was affected more often than any other organ, the frequency was not so great as to be significant.

Exper. 2.—To control experiment 1 four strains of colon bacilli isolated from the gastrointestinal canal of rabbits were injected into 3 rabbits. Not a single lesion was produced in the kidneys of these 12 animals. Only one showed localization of any kind; purulent appendicitis was developed in this case.

Exper. 3.—Varying the technic by chilling the animals shortly after intravenous injection of the colon bacillus increased the incidence of renal lesions to 5 in 20, in animals given injections with organisms isolated from the urine of a patient with pyelitis. In eight animals there was other localization also. Cholecystitis was found in five, ulcer of the stomach in one, appendicitis in one, and acute metritis in one. Thus the incidence of lesions in the kidney was twice as great as in experiment 1 without a corresponding increase in lesions elsewhere.

Comment.—In experiment 2 there was no renal localization of the colon bacillus isolated from the gastrointestinal tract, whereas the eight strains isolated from the urine of patients with pyelitis localized in the kidney in about 12% of the animals in experiment 1. These control and double-control experiments indicate the tendency of colon bacilli isolated from the gastrointestinal tract of rabbits and from the urine of patients with pyelitis to produce local lesions, but without any specific tendency to select a special organ.

A fairly comprehensive search of the literature has not revealed any report concerning the production of renal lesions with any degree of

constancy by intravenous injection of colon bacilli, when there was no obstruction of the urinary outflow. The discovery of spontaneous pyelitis in a female rabbit caused by *Bacillus coli communior* that showed a specific tendency to produce local renal lesions when injected intravenously into rabbits, was, therefore, of great interest both in regard to the production of renal lesions with a colon bacillus and the specific tendency of an organism to produce local lesions.

The pathologic changes in the kidneys of this rabbit indicated pyelonephritis caused by a colon bacillus, a condition hitherto undescribed in the rabbit. The kidneys were about normal size, smooth, and reddish brown. Both ureters were dilated up to the brim of the pelvis. The pelves of the kidneys were distended, the left with thin purulent material. There were no abscesses on the surface of the kidneys or on section.

The histologic changes were alike in both kidneys, only more marked in the left. The outstanding feature was the extensive intertubular infiltration with polymorphonuclear leukocytes most marked in the medulla but extending up into the cortex. In the right kidney the glomeruli were full of eosinophilic leukocytes. The pelvic lining was not inflamed in any of the sections examined. There was none of the sclerosis which accompanies the focal nephritis so frequently seen in the rabbit.

Exper. 4.—*Bacillus communior* isolated in pure culture from the renal pelvis of this rabbit, was injected into 32 rabbits. The organisms in 15 cc. of a 24-hour culture in dextrose brain broth were separated by centrifugation and suspended in 5 cc. of a solution of sodium chloride. One cc. of the suspension was injected into the vein of the ear. Of these animals, 22 showed renal lesions, 16 having visible abscesses of the kidney and 16 definite pyelitis. In ten both lesions were observed. Recently we learned that even with abscesses in the cortex of the kidney the urine may be sterile (fig. 1). Only three animals showed a local lesion outside of the urinary tract. One animal had an abscess of the seminal vesicle, one, hemorrhage into the mucosa of the cecum, and one, purulent peritonitis with severe coccidiosis.

Comment.—The localization of this strain of colon bacillus in the kidney was striking when compared with the results in experiment 2 in which renal localization was not found and also with the results in experiment 1 in which localization occurred in only 12% of the animals. It might be argued that this organism from spontaneous pyelitis was

more virulent and, therefore, tended to produce lesions more extensively, were it not for the fact that in experiment 4 there were only three instances of localization outside of the kidneys whereas in experiment 1 there were 21 such instances. Extrarenal lesions occurred in experiment 1 in 32%, in experiment 2 in 8%, and in experiment 4 in 9%.

A summary of this series of experiments shows that local renal lesions occurred in 22 of 32 animals given intravenous injections with colon bacilli isolated from the urine of a rabbit suffering from spontaneous pyelitis. Twelve control rabbits injected with four different strains of colon bacilli isolated from the gastrointestinal tract of rabbits did not have renal lesions. Colon bacilli isolated from the urine of patients with pyelitis localized in the kidney in 8 of 66 animals that were given injections.

From 1921, when we resumed our studies, to 1925, we were constantly on the watch for an organism that would regularly produce renal lesions when injected intravenously into rabbits. Injections were made in from two to four animals with almost every strain of colon bacillus isolated from the urine of patients with pyelitis. Although a considerably larger number of animals were used, records were kept of only 49; in seven of these there was renal localization.

Expcr. 5.—In 1925 it was found that an organism isolated from the urine of an infant dying from pyelitis would localize in the kidney of rabbits. This organism was used to produce pyelitis, and the latter was treated with hexylresorcinol, methenamine, and mercurochrome.⁶

In one group of 25 rabbits (table 1) every animal developed pyuria, and definite cortical or pelvic lesions were found at necropsy. A number were cured but the urine still contained numerous pus cells. The infection in one set of four untreated control animals cleared up in about 30 days.

Expcr. 6.—After an interval of a month, in spite of passage through animals and keeping the organism in various mediums in the ice chest, its virulence was so reduced that not a single renal lesion developed in the 20 animals to which injections were given.

Expcr. 7.—The specific tendency of some strains of colon bacilli to produce renal lesions is perhaps most clearly seen in the experiment in which colon bacilli isolated from the urine of a rabbit with spontaneous

⁶ Helmholz, H. F.: J. Urol., 1922, 8, p. 301. Helmholz and Beeler: Am. J. Dis. Child., 1917, 14, p. 5. Helmholz and Field: J. Urol., 1924, 15, p. 351. Helmholz and Rappaport: Arch. Pediatrics, 1917, 34, p. 658.

pyelitis were injected. This organism was isolated in November, 1926, and persisted in the rabbit's urine until March 28, 1927 when the animal was killed.

The animal was anesthetized with ether and both ureters were tied two hours before necropsy, at which extrarenal organs were found free from lesions. The bladder was practically empty, and did not show perivesicular edema or redness or thickening of the lining. The ureters were slightly dilated above the obstruction. Urine from the right

TABLE 1
EXPERIMENTAL PYELITIS BY INTRAVENOUS INJECTION

Rabbit	Weight, Kg.	Virulent Bacillus eoli Injected, Cc.	Days Animal Lived after Injection	Catheterized Specimen		Specimen at Necropsy		Abscesses in Kidney
				Cul- ture	Pus, Grade	Cul- ture	Pus, Grade	
1	2.15	1	15 (killed)	+	4	+	4	—
2	1.5	1	14 (died)	0	4	0	4	0
3	1.5	1	2 (killed)	—	—	+	4	+
4	1.9	2	2 (killed)	—	—	+	4	0
5	—	1	3 (killed)	—	—	+	4	0
6	—	1	14 (killed)	+	1	0	—	0
7	2.0	1	2 (killed)	—	—	+	4	0
8	2.0	2	8 (died)	+	4	+	4	+
9	1.25	1	11 (died)	+	4	+	2	0
10	1.3	1	17 (killed)	+	4	+	4	0
11	2.3	1	20 (died)	+	4	+	4	+
12	1.25	1	13 (died)	+	4	+	4	+
13	1.75	1	16 (killed)	+	4	+	4	+
14	2.1	1	14 (died)	+	4	+	4	+
15	1.85	1	20 (died)	+	4	+	4	+
16	1.25	2	14 (killed)	+	4	+	4	+
17	1.25	2	12 (died)	+	4	+	4	+
18	2.15	2	34 (killed)	+	4	0	4	+
19	1.95	2	34 (killed)	+	4	0	1	+
20	1.3	2	34 (killed)	+	4	0	1	0
21	1.8	2	28 (killed)	+	4	0	3	0
22	2.15	2	33 (killed)	+	4	0	0	+
23	1.4	2	33 (killed)	+	4	0	3	+
24	1.95	2	33 (killed)	+	4	0	0	+
25	1.25	2	33 (killed)	+	4	0	2	+

In all tables + indicates presence of lesions or bacteria, and 0, their absence; — indicates no test made.

ureter was yellow and contained large masses of pus. Urine from the left ureter was blood-stained but pus was not visible. The kidneys were pale brown with scattered small depressions on the surface. There were no signs of acute abscess. On section there was a discharge of purulent material from the right pelvis but otherwise the kidney appeared normal, as did the left kidney. Pure cultures of colon bacilli were obtained from both pelves.

Histologically, the mucous membrane of the bladder showed definite areas of infiltration with both polymorphonuclear leukocytes and lymphocytes. In one area deep in the muscle layer there was a group of blood vessels crowded with polymorphonuclear leukocytes. The

histologic changes were much alike in both kidneys, but more marked in the right. In both there were chronic as well as acute changes. The changes in the cortex resembled closely those found in some cases of spontaneous nephritis in which there is a marked tendency for the glomeruli to escape the effects of the process. There was no acute reaction in the cortex. In the medulla there was focally considerable increased fibrous tissue in one area just beneath the pelvic lining. The tendency of the vessels to be filled with polymorphonuclear cells was marked only close to the angle in the pelvis near the base of the papilla. The pelvis showed both chronic and acute changes. No section has shown such dense infiltration of the mucosa (fig. 2). The serosa was thickened and the subserous tissues infiltrated with leukocytes and lymphocytes and with an excess of connective tissue cells. The acute process was limited to the pelvic and peripelvic portions of the kidneys.

TABLE 2
NECROPSY DATA ON ANIMALS IN EXPERIMENT 7

Series (10 Animals in Each)	Infected	Number of Animals					
		With Infected Kidneys				With	
		Gross		Microscopic		Infected Urine	
		Pyelitis	Abscess	Pyelitis	Abscess	Bacteria	Pus
1.....	4	—	1	—	—	2	3
2.....	6	2	3	3	2	7	5
3.....	5	1	1	1	2	5	4
4.....	7	6	6	6	5	8	6
5 (13 animals)....	12	3	8	5	6	13	10

Of the 53 animals used in this experiment, 34 showed definite evidence of localization of colon bacilli in the urinary tract. There was considerable variation in the percentage of positive renal infections encountered. By passage through animals the organism definitely acquired greater power of producing local renal lesions. If the experiment is divided according to the time of injection into four series of ten animals each and one of thirteen animals, the percentage of positive results was 40% in the first series, 60% in the second, 50% in the third, 70% in the fourth, and 92% in the fifth (table 2). That virulence may increase to the lethal point was shown recently when animals injected with smaller amounts of cultures from the same organism died from toxemia in a short time without local symptoms. As was observed in some previous experiments there seems to be a tendency toward spontaneous recovery in from 30 to 40 days. In 3 of 8 animals that lived more than one month the urine was sterile and free from pus at necropsy, although organisms and pus cells were present only a few days before the animals were killed (table 3).

The most striking changes were in 5 animals that were killed by chloroform 24 hours after injection (table 4). In four, acute small yellow abscesses, 1 to 2 mm. in diameter, were found in the cortex of the kidneys (fig. 3). Section demonstrated that some extended deeper into the cortex and even into the medulla, although deep abscesses were more common in the animals killed after a longer period. In all five,

TABLE 3
STERILITY OF URINE IN ANIMALS OF EXPERIMENT 7 LIVING 30 TO 40 DAYS

Rabbit	Days Lived	Days Urine Contained		Necropsy Data			
				Kidney		Urine	
		Bacteria	Pus	Pyelitis	Abscess	Bacteria	Pus
1.....	36	30	25	0	0	0	0
2.....	37	—	—	0	0	0	0
3.....	32	32	32	0	+	+	+
4.....	35	35	35			+	+
5.....	30	30	30	+	0	+	+
6.....	43	43	43	0	0	+	+
7.....	31	28	28	0	0	0	0
8.....	31	28	28	0	0	0	0
9.....	34			0	0	0	0

TABLE 4
NECROPSY DATA ON RABBITS KILLED AT DIFFERENT INTERVALS AFTER INJECTION

Group	Number of Rabbit	Kidney		Urine	
		Pyelitis	Abscess	Bacteria	Pus
5 rabbits, killed 24 hours after injection	1	0	+	+	+
	2	0	+	+	+
	3	0	0	+	0
	4	0	+	+	0
	5	0	+	+	+
10 rabbits, killed 48 hours after injection	6	0	0	0	0
	7	0	0	0	0
	8	0	0	0	0
	9	0	+	0	0
	10	0	0	+	0
	11	0	+	0	0
	12	+	+	+	+
	13	+	+	+	+
	14	+	+	+	0
	15	0	0	0	0

innumerable bacteria were present in the urine, but only in three was pus present in definite amounts. In none was pus visible in sections of the pelvis.

Only 5 of the 10 animals which were allowed to live for two days after injection showed abscesses in the kidney (fig. 1), and in two of these, cultures of urine at necropsy were negative (table 4). In one of the five animals which did not show abscesses, cultures of the urine were positive. In contrast to the results in the animals that lived for one day, there were three animals in the 2-day group with definite amounts of pus in the pelvis of the kidney.

In the kidneys of the animals which lived for a short time there were usually small yellow abscesses in the cortex, extending in yellow streaks down into the medulla, and even into the pelvis, which was always definitely involved 48 hours after injection. On the other hand the kidneys of animals which were allowed to live longer showed relatively few large abscessed areas (fig. 4). Whether the greater number heal and only a few continue to develop cannot be determined until we have operated on a series of animals, viewed the kidney 48 hours after injection, and then allowed the animals to live for several weeks longer. The cortical abscesses are the most striking pathologic feature of the kidneys of the animals living for a short time, whereas in the kidneys of animals living longer pyelitis only may be present (fig. 5). In one rabbit (exper. 7) which lived for 16 days, evidence of abscesses was

TABLE 5
SUMMARY OF EXPERIMENTS

Specific Localization			Controls		
Experi- ment	Number of Animals		Experi- ment	Number of Animals	
	Receiving Injections	Showing Renal Lesions		Receiving Injections	Showing Renal Lesions
4.....	32	22	1.....	66	8
5.....	25	25	2.....	12	0
7.....	53	34	3.....	20	5
			6.....	20	0
			From 1921 to 1925..	49	7

not found and the pelves of both kidneys were filled with thick, creamy, yellow pus. Three animals constantly passed urine which contained pus and innumerable colon bacilli for a period of four weeks after injection, and yet at necropsy the urine did not contain pus, and cultures were sterile. The kidneys of these animals did not show definite evidence of pyelitis or local cortical lesions in the sections examined. Serial sections were not made.

In this experiment extrarenal involvement was exceptional. In only three animals were there local lesions in the bowel; these were manifested principally by hemorrhagic areas in the small bowel and colon. Involvement of the gallbladder was difficult to ascertain because of the marked coccidiosis. None of the animals showed definite cholecystitis with external exudates in the gallbladder as seen in experiments 1 and 3. In one instance there was acute peritonitis apparently secondary to a large renal abscess. This, however, may have been from some other focus not found at necropsy but in an animal that had lived several weeks after injection it was certainly not a primary involvement.

DISCUSSION

The relation of these results to the problem of producing local infection in the kidney with the colon bacillus is apparent. In ten years we have tried out a vast number of organisms but, unfortunately, we did not keep track of all the results as we were not primarily interested in the problem of specific localization but merely wished to produce renal lesions with sufficient regularity to study their development and healing and the therapeutic value of various urinary antiseptics. Of the large number of cultures injected three gave striking results (table 5). Renal lesions were produced in 66% of the animals given injections in experiment 4, in 100% in experiment 5, and in 66% in experiment 7. In none of these experiments was there corresponding increase in the extrarenal lesions. This alone is sufficient to mark the organisms as of unusual nature. As controls we have organisms isolated from cases of pyelitis in man and organisms isolated from the urine of rabbits which show no tendency to localize in the kidney; the former show a tendency to localize elsewhere.

SUMMARY

In studying various strains of colon bacilli isolated from the urine of patients with pyelitis, from the urine of rabbits, and from the gastrointestinal canal of rabbits, three strains showed a specific tendency to produce local renal lesions. More than two-thirds of the animals given injections with these strains showed renal localization without corresponding increase in localization elsewhere. This is the first report of the specific localization of the colon bacillus.

PLATE 1

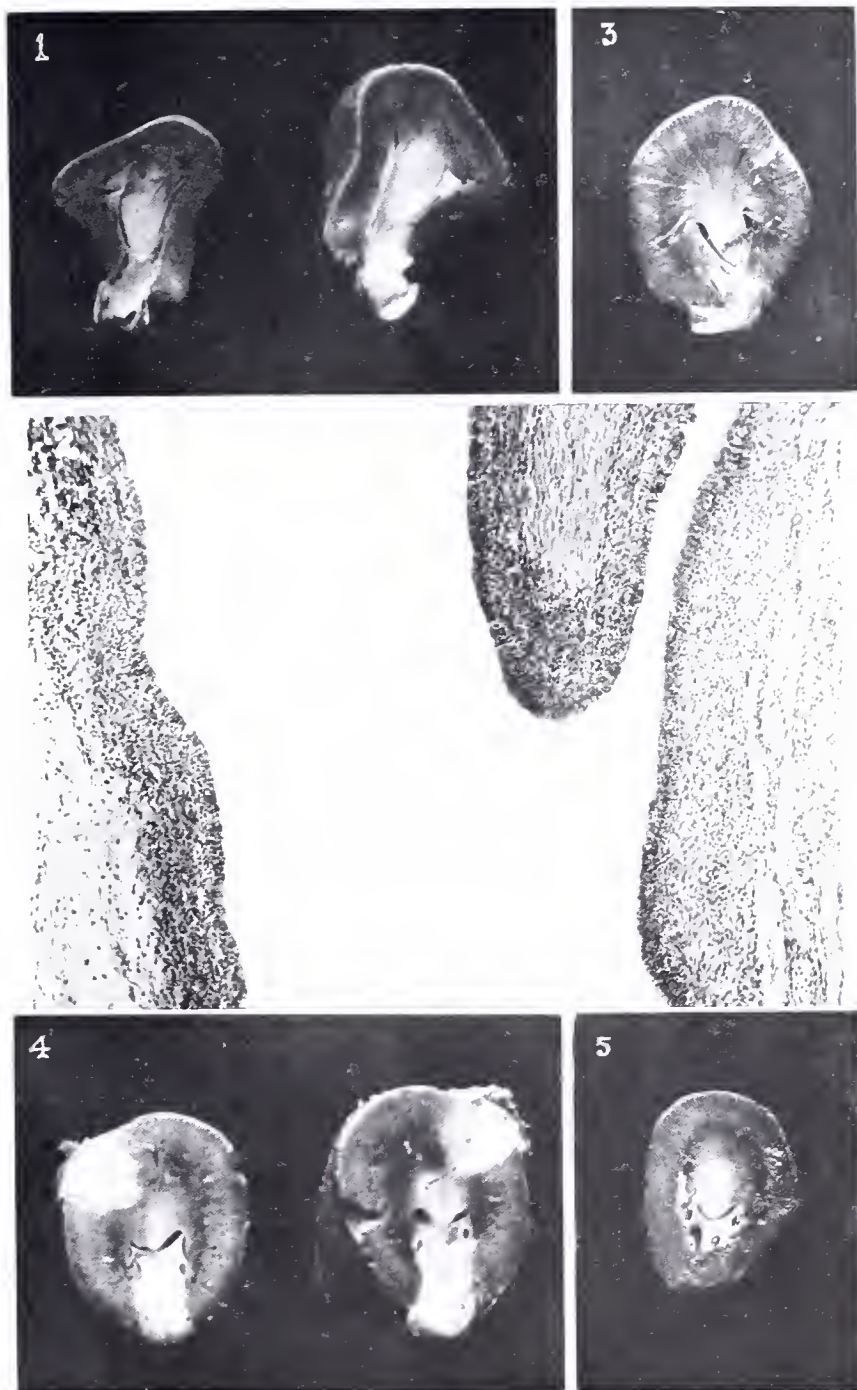


Fig. 1.—Abscesses in the renal cortex; urine cultures negative.

Fig. 2.—Chronic and acute inflammation of kidney. Spontaneous pyelitis ($\times 60$).

Fig. 3.—Cortical and medullary abscess found at necropsy 24 hours after injection of colon bacilli.

Fig. 4.—Large, chronic cortical abscess.

Fig. 5.—Pyelitis without renal abscesses.

NONSPECIFIC WASSERMANN AND AGGLUTININ REACTIONS WITH SERUMS FROM PATIENTS WITH FEBRILE DISEASES

MARION CORRIGAN

From the Otto Baer Fund for Clinical Research of the Michael Reese Hospital and the Nelson Morris Institute for Medical Research, Chicago

In the early days of the routine use of the Wassermann reaction, malaria, pneumonia, scarlet fever and a few other conditions associated with fever were reported as giving falsely positive reactions in an indefinite proportion of instances. Although the standardization of the reaction has reduced the number of false results, the value to be attached to a positive Wassermann reaction in diseases with high temperatures is questioned, to the extent of eliminating the routine Wassermann test in such cases in many hospitals. Roaf¹ thinks it probable that a transient Wassermann reaction may be a constant phenomenon during the acute stage of relapsing fever. He found six of nine cases which were positive at some stage of the disease, to be negative 8 to 13 days after the onset of the fever, and two cases, which were negative on first examination, to be positive 3 to 6 days after the onset of the fever. Fairley and Sullivan² state that during the pyrexial period, in both benign and malignant tertian fever, it is not uncommon for three units of complement to be fixed. Kolle and Hetsch³ observed positive Wassermann reactions with serums of nonsyphilitic persons, violently ill, at the point of death. They consider that under agonal circumstances alterations of the serum take place, which affect the Wassermann test, but they likewise suggest the possibility of latent syphilis as the cause of these unanticipated reactions. The serums of patients with scarlet fever or with pneumonia before the crisis have been regarded with special disfavor. Christensen⁴ has reviewed the subject in relation to scarlet fever, and from his own experiences decided that though weakly positive reactions occur as the result of scarlet fever, they are not of diagnostic significance.

Received for publication, Sept. 27, 1927.

¹ Brit. J. Exper. Path., 1922, 3, p. 59.

² J. Roy. Army Med. Corp., 1919, 33, p. 268.

³ Die Experimentelle Bakteriologie und die Infektions Krankheiten, 1911, p. 652.

⁴ J. Am. M. A., 1923, 80, p. 1118.

Green ⁵ found the Wassermann reactions with serums of febrile patients to be negative.

Because of occasional discrepancies in either the Wassermann or the agglutinin reactions of serums of patients with high temperatures, the occurrence of nonspecific serum immune reactions and the persistence of these after temperature had subsided, was investigated in a series of such febrile cases. The Wassermann test was made, and agglutinins and complement-fixing antibodies for *B. typhosus* and *B. proteus* X19 were determined. *B. proteus* X19 antibodies were investigated because these had been occasionally observed in the serums of patients with diseases other than typhus, and if nonspecific reactions were to occur, it was thought probable that they would be of a proteus-fixing variety.

Tests were made on the serums of patients whose temperatures were 103 F. and over. The 100 cases included pneumonia, typhoid fever, febrile heart cases, septicemia, meningitis, local infections, and a miscellaneous group consisting of uremic coma, carcinoma of the uterus, peritonitis, malaria injection, postencephalitic Parkinson's syndrome, scarlet fever, erysipelas, acute rheumatic arthritis, typhus, aplastic anemia, suppurative arthritis, spasmophilia, acute catarrhal jaundice and pyelitis.

Three antigens were used in the Wassermann test; a cholesterol antigen incubated at 37.5 C., a lipoid and a luetic liver antigen both incubated at 4 C. Salt solution suspensions of 18-hour cultures were used as antigens in the bacterial complement-fixation tests. The agglutinin titers of the serums were determined by macroscopic agglutination tests.

From 45 febrile patients with pneumonia, 11 serums (24%) gave positive reactions of one kind or another. Of these, three, giving positive Wassermann reactions, were from patients with a history of having had syphilis. One serum gave a three plus reaction with lipoid and luetic liver antigens (4 C. incubation) and a negative with cholesterol (37.5 C. incubation). No history of syphilis could be obtained and the patient died before control tests could be made. The serum from one patient gave a four plus fixation on two occasions before the crisis and a negative reaction after the crisis and fall of temperature. Hertz ⁶ describes ten cases of pneumonia in which positive reactions occurred between the fifth and eight days of the disease and negative reactions after the tem-

⁵ J. Lab. & Clin. Med., 1923, 9, p. 80.

⁶ Proc. New York Path. Soc., 1915-16, n.s. 15, p. 152.

perature had fallen to normal. Of the positive results with *B. typhosus* and *B. proteus* X19, three were of diagnostic strength. A 1:40 agglutination of *B. typhosus* and a two plus fixation with *B. typhosus* became negative when the temperature had subsided. A 1:80 agglutination of *B. typhosus* was obtained with the serum of a patient from the U. S. Veteran's Bureau Hospital, who had undoubtedly been immunized at some time. The reactions observed in the other three cases were of nonspecific nature, often encountered in the serums of normal individuals and of insufficient strength (1:10 or 1:20) to be of diagnostic significance.

In 5 cases of meningitis and 8 cases of local infections, the serums gave only negative results. Eight cases of typhoid, giving strongly positive typhoid agglutinations and complement fixations, gave negative Wassermann and *B. proteus* reactions.

In one of nine febrile heart cases a positive result occurred. This was a case of subacute bacterial endocarditis in which there was a four plus fixation with cholesterol antigen. No other indications of syphilis could be detected. This positive result was obtained simultaneously with a blood culture of *Streptococcus viridans* and may be the type of finding reported by Storp,⁷ who is of the opinion that the coincidence of positive blood cultures and positive Wassermanns is not accidental and that the abatement of the disease and the disappearance of bacteria from the blood go parallel with the disappearance of complement-fixing antibodies. On the other hand, a case of subacute ulcerative endocarditis, yielding a blood culture of *Streptococcus viridans*, simultaneously gave a negative Wassermann reaction.

Six cases of septicemia, with but one exception, gave negative results. This one gave a positive agglutination of *B. typhosus* in dilution of 1:40, which persisted after the fall of the temperature to normal, although there was no history of typhoid or of vaccine administration. Storp⁷ reports a case of staphylococcus septicemia, in which, on three occasions, a four plus Wassermann reaction was obtained, followed by a complete negative six days after the temperature had subsided. There was neither history nor other indication of syphilis, so the positive was considered to be false. The same author reports similar results with the serums of patients having high temperatures in pneumonia and streptococcus infections.

⁷ Deutsch. med. Wchnschr., 1923, 49, p. 1014.

In the miscellaneous group two cases of typhus showed agglutinins for *B. typhosus* in dilutions of 1:40 and 1:50, which became negative on the abatement of the temperature. On the other hand, serum from a patient suspected of having typhus fever agglutinated *B. proteus* X in dilution of 1:640 on two occasions; typhus fever was later ruled out as a diagnosis. A two plus Wassermann reaction with cholesterol antigen was obtained with the serum of a patient having no history of syphilis but symptoms of epilepsy.

SUMMARY

With serums from 100 patients with febrile diseases, two strongly positive Wassermann reactions were unaccountably obtained in cases of pneumonia with no syphilitic histories, and in one of these the Wassermann reaction became negative after the crisis; one strongly positive reaction was observed in a nonsyphilitic case of endocarditis; and a weakly positive reaction (two plus with cholesterol antigen) occurred in a case of epilepsy. A 1:40 agglutination and a two plus fixation with *B. typhosus* occurred in two cases of pneumonia without history of typhoid immunity. The serum of two patients with typhus agglutinated *B. typhosus*. The serum of a patient who proved not to have typhus agglutinated *B. proteus* X in a dilution of 1:640.

Reactions of an apparently false nature were thus observed to occur with serums of patients with high temperatures, but on so few occasions that a causal relationship of the temperature may be doubted. The temperature on the other hand may be instigative of a latent luetic condition or of the fluctuation of nonspecific agglutinating antibodies. In the latter case antityphoid vaccination or recovery from a mild attack of typhoid cannot be excluded with absolute certainty.

Nonspecific reactions occur so infrequently that their possible occurrence should not bar the routine use of the Wassermann or other serologic reaction for diagnostic purposes.

ON THE BIOLOGY OF *B. FUSIFORMIS*

JOSEPHINE S. PRATT

From the Pathological Laboratory of the Fifth Avenue Hospital, New York

The almost constant presence of the fusiform bacillus in broth cultures inoculated from swabs taken from the throats of a group of individuals with tonsilitis and common colds was so striking that it was decided to isolate and study these bacilli. A review of the literature showed that very little had been done on pure cultures of the group, and nothing on the serological relations.

The earliest writers report the finding of a spindle shaped bacillus and note only its oxygen requirements, the production of a foul odor and slight pathogenicity. Weaver and Tunnicliff¹ in 1905 isolated a fusiform bacillus in pure culture but confined themselves to a study of its morphology and pathogenicity. In 1912, in association with Krumwiede² I studied fifteen strains from noma, gangrene and various mouth conditions. We found no constant differences in morphology among our strains, and that the only definite basis for grouping was the ability of some to ferment sucrose, a difference which had no relation to the source of the strain. Klimenko³ in 1914 studied several strains but gave no cultural or immunological details. He found that his cultures had a slight pathogenicity, which he believe grew less as the strain aged. Dick and Emge⁴ in 1914 isolated a fusiform bacillus from a brain abscess shortly before the death of the patient. They gave no cultural characteristics, but found they could produce a paralysis and chronic convulsions when the freshly isolated culture was injected into the meninges of a rabbit. Some time later they repeated the experiment with negative results. Mellon⁵ in 1919 isolated a fusiform bacillus from a case of pyemia. His report dealt merely with morphology. In 1923 Tunnicliff⁶ reported the cultural reactions of one strain. She stated that this strain required body fluid for growth, though small amounts sufficed, and that it fermented dextrose, lactose, salicin, inulin, but not maltose or sucrose. Knorr⁷ in 1922 isolated several strains which he thought fell into two morphological groups but gave no details of cultivation. Varney⁸ in 1927 isolated eighteen strains, classifying them into four groups, based on morphology and agglutination reactions. There was no correlation between these groups and the source of the cultures.

That small numbers of fusiform bacilli are normally present in the throats of men and of certain animals is shown by the fact that fusiform bacilli were found in the mixed broth cultures made from the throats

Received for publication, Oct. 13, 1927.

¹ J. Infect. Dis., 1905, 12, p. 446.

² Ibid., 1913, 13, p. 438.

³ Centralbl. f. Bakteriöl., I, O., 1914, 74, p. 487.

⁴ J. Am. M. A., 1914, 62, p. 446.

⁵ J. Bact., 1919, 4, p. 505.

⁶ J. Infect. Dis., 1923, 33, p. 147.

⁷ Centralbl. f. Bakteriöl., I, O., 1922, 89, p. 4.

⁸ J. Bact., 1927, 13, p. 275.

of more than 200 human beings, in 9 of the cultures from 15 rabbits, in 12 from 18 guinea-pigs, and 3 from 3 monkeys. Included in the series for human beings were persons with common colds, lung abscess, pneumonia, Vincent's angina, tonsilitis, various abnormal mouth conditions and about 50 persons with no evidence of infections of the mouth or upper respiratory tract. Frequently the bacilli may be found in direct smear from the throats of normal persons, and more often in chronic sinusitis, enlarged tonsils, or some form of oral infection.

It is a very simple matter to demonstrate the presence of the fusiform bacillus, quite another thing to isolate it and, having isolated it, to keep it alive. If the material collected on a swab is washed off in infusion broth, preferably without ascitic fluid, the fusiform bacillus, if present, will multiply sufficiently in 24 hours to develop its odor and to be found in smear. It is not necessary to employ any special procedures for anaerobiasis. It seems probable that the aerobic organisms help the growth of the fusiform by using up the oxygen present in the medium, and perhaps as they die off they furnish food. Certainly association with these organisms is a factor, for the medium used for pure cultures must be freshly made and used before it has had time to absorb much oxygen. Moreover the growth in pure cultures appears to be less vigorous than in mixed. Transplants of mixed cultures can be made, however, only a limited number of times, for a time will be reached when the fusiform bacilli disappear and only the contaminating bacteria develop.

Dilutions great enough for proper spacing of colonies often went beyond the point where the bacilli were present, while lower dilutions resulted in plates too crowded with contaminating bacteria for isolation. It was hoped that mixed cultures might be purified by the use of one of the aniline dyes. Gentian violet, brilliant green and a mixture of the two were tried with indifferent success. After incubation for 24 hours the dye or (dyes) in the proportion of 1:5000 of ascitic broth, was added to the sediment and the tube reincubated. In some cases there appeared to be inhibition of the other bacteria, in others the dye seemed to have no effect, and the results on the whole so variable that the use of dyes was discontinued.

Two methods of dilution gave good results. In one, 6 tubes, each with 0.5 cc. ascitic fluid, were set up. To the first, about 0.05 cc. of the sediment of a 24 hour impure culture was added. After mixing, about 0.05 cc. was transferred to another tube, repeating this through the

series. The contents of tubes 4, 5 and 6 were plated. Better results were obtained when a 1:50 dilution of the sediment was made in broth and one loop, (4 mm.) of this was transferred to 0.5 cc. of ascitic fluid. From this second tube one and two loops were transferred to 0.5 cc. ascitic fluid. These dilutions were made and plated in duplicate. The method devised by Krumwiede and Pratt² of inverting the bottom of a petri dish in the cover was used. Sufficient beef infusion agar, at least 8 cc., to insure a good seal at the rim is necessary for successful plating. Plates were incubated 44 hours, then opened by wrenching the cover off, and the colonies selected under the microscope, using a number 2 lens. The colony is characteristic but not individual, that of several other anaerobes resembling it. It is rather large, flat, irregular in outline, with a tuft near the center, and has a greenish metallic sheen. Occasionally one finds a colony which is wedge shaped with the tuft sticking out at the side.

For isolation, the medium of choice is dextrose agar (0.25% agar) with 0.5% ascitic fluid. The soft agar is to be preferred to a stiffer one as growth takes more rapidly and transplanting is much easier. Less enriching fluid was used than is the general practice, when the work was begun. Later, larger amounts were used with no better results, so we returned to the small quantity. The best development was in mediums with a P_H of 7.6. It is wise to inoculate 15 or 20 tubes from individual colonies as the percentage of successful growths from plates is painfully low. Stock cultures may be maintained in this dextrose agar with ascitic fluid, transplanting at least once in two weeks. Transplants are made with a pipet, expelling the growth at the bottom of the freshly prepared tube. Care must be taken not to introduce any air bubbles. Growth will appear only at the bottom and will not extend beyond a quarter of an inch from the top of the medium. The colonies are fluffy, and while there may be a dense amorphous mass of growth, there are usually puffball masses scattered throughout. The growth of pure cultures is less vigorous in broth than in agar and appears only at the bottom of the tube in a soft fluffy mass. Mediums must be boiled shortly before needed, and used before they have had time to reabsorb oxygen. Inoculations in agar may be made while it is still soft. During the course of this study 20 strains were isolated, but unfortunately only 9 survived for complete tests, some dying out rather promptly, others after longer intervals.

The fusiform bacillus is an obligate anaerobic, gram-negative, non-motile rod, generally pointed at both ends and with one or more granules. My strains fell into two morphologic types, which may be differentiated in young cultures, one, short and sharply pointed at both ends with a somewhat swollen central portion with one granule, the other longer with tapering or sometimes blunt ends, more regular width and two central granules. In older cultures these differences disappear and only long filaments with granules are found. The bacilli stain with any of the ordinary dyes, especially with carbol fuchsin diluted 1:10 and heated gently, while the granules are brought out with any of the polychrome stains. In routine work the smear was fixed with methyl alcohol, and flooded with aqueous eosin 1:1000 for three minutes, then the stain washed off with azure 1, 1:400 and differentiated for three minutes. The viability of *B. fusiformis* is very variable. Some strains died out after the fourth or fifth transfer, others have been in cultivation for a year. Transferring once in two weeks seems safe, though some cultures were viable after four months. All workers, so far as I know, state that these organisms require body fluid for growth. This is undoubtedly true of pure cultures in the early generations, but I believe that it does not hold for mixed cultures or for old strains. As has been said, the best results in enriching the organisms were obtained when inoculations were made in beef infusion broth without ascitic fluid. I was able to get good growths for ten generations in 0.5% beef infusion agar without body fluid or added carbohydrate, using two 6-month-old strains. It is certain that some ascitic fluid was carried over into the first sub-culture, and possible that there was enough in the second to aid growth, but I believe that beyond this point the amount of fluid transferred was negligible. The growth in the tenth generation, when the experiment was stopped, was as good as that in the second. For comparison duplicates were made in agar from the same batch of which 1% dextrose and 0.5% ascitic fluid were added. I could detect no difference in the amount of growth in the two mediums.

Fermentation reactions were tested with dextrose, lactose, sucrose, mannitol, maltose and salicin in sugar free infusion agar (0.5%) containing 1% Andrade indicator, but without ascitic fluid. All strains fermented dextrose, four fermented both dextrose and sucrose. One, a strain from a case of Vincent's angina which developed a fatal lymphatic leukemia, fermented dextrose, sucrose and salicin. One strain from a rabbit fermented dextrose, sucrose, mannitol, maltose and salicin. The

production of gas was never noted. In general these results confirm those of Krumwiede and myself² in separating the group into two divisions, one fermenting sucrose, one not, with no correlation between this difference and the source of the culture. It is interesting to note that the strain having the most active fermentative capacity is of rabbit origin, though even here there is no uniformity, for the other rabbit strain tested fermented only dextrose.

The results of animal inoculation will be reported elsewhere. The fusiform bacillus seems to have some pathogenicity, but it is variable and quickly lost. Attempts to demonstrate a soluble toxin were unsuccessful.

Complement fixation was selected as the most feasible method of determining the serologic relationships. Agglutination was not considered because of the character of the growth in broth which was the source of the mass cultures. Attempts were made to test the precipitin reaction, but the antisera available gave no precipitate with the extracts prepared. Rabbits were given intravenous injections at intervals of 3 days with 1 cc. of the centrifugated sediment of 24-hour living broth cultures, 10 to 15 injections being given. The animals were bled on the 4th day following the last dose. Evidently the fusiform bacillus has but slight antigenic power, as serums of only low titer were obtained. For preparation of the antigen, 50 cc. of the dextrose beef infusion broth were boiled to expel the oxygen, cooled to 40 C., inoculated, and covered with paraffin. Incubation was for one week. The cultures were centrifugated, and the sediment treated with alcohol and ether, according to Wilson.⁹ Some did not dry completely, so dilutions were made from the moist sediment. The resulting antigens seemed as satisfactory as those made from the dried material. Some of the antigens were quite strong, giving fixation in a dilution of 1:75, but most of them were weaker, and were used undiluted. None was anticomplementary. Three serums were tested with six antigens. In no case was there fixation of any degree with a heterologous antigen, but fixation was always complete with the homologous antigen. These results are another support of our belief that the group is a heterogenous one, with no relationship among the members.

Some observers, notably Tunnicliff, consider that the fusiform bacillus and spirochete which commonly accompanies it are different forms of the same organism. Other observers, including Krumwiede and

⁹ Park and Williams: *Pathogenic Microorganisms*, 1924, p. 273.

myself,² when we worked on the group 1912, have disagreed with this view, nor has the work I have recently done made me change my opinion. In an effort to get more precise information on this point, freshly made serum water medium to which a piece of sterile guinea-pig kidney was added, was put in tubes (200 mm. \times 15 mm.) and inoculated. Two strains were used, one of the long variety which had been in cultivation for six months, and one, of the short morphologic type, one week old. At the same time these strains were also inoculated into the soft dextrose ascitic agar used in routine work. Smears and dark field examinations were made after 2, 4, 7, 14, 21 and 30 days' incubation. At no time was any motility noted, or any forms which could be confused with true spirochetes found. As the cultures grew older the differences in morphology were lost, long thread like forms being found in all transplants. These bacilli often stretched over several fields. Granules were present at regular intervals, and the body of the bacillus, which in young cultures was markedly basophilic, stained a faint lavender, often appearing only as a shadow. Some of these long forms were wavy, but the waves were large and irregular, with no resemblance to those of a spirochete. After a careful study of these smears I am convinced that the fusiform bacillus and the spirochete are two separate and distinct species. Varney, who has made a painstaking study of these bacteria does not believe that the spirochete and the fusiform bacillus are different forms of the same organism. In some of the strains he found loose wavy threads as I did, and states that while they resemble spirochetes, he believes they are not identical. The cultures isolated by me seem to agree with his types 1 and 2. In my cultures I have seen variations of both types which resemble his type 3. Type 4 I have never seen.

CONCLUSIONS

Fusiform bacilli seem to be present normally in small numbers in the throat; being found in 100% of the human beings examined, in 100% of the monkeys, in 66% of the guinea-pigs and in 48% of the rabbits in this series. Under certain abnormal conditions these bacteria increase and probably function as secondary infecting agents. On the basis of sucrose fermentation two cultural groups are demonstrable, but the difference bears no relationship to the source of the cultures. Complement fixation failed to prove the existence of serological groups, each antigen fixing its homologous antiserum only, indicating a serologic heterogeneity. The fusiform bacillus and the spirochete commonly found together are different microorganisms.

THE PROTEASES AND ANTIPROTEASES OF PLEURAL EXUDATES

CHARLES WEISS*

From the Departments of Bacteriology, College of Physicians and Surgeons, Columbia University, New York, and the School of Tropical Medicine, University of Porto Rico, San Juan, Porto Rico

It has been observed recently by Gay and Clark¹ that a mixture of aleuronat and starch injected intrapleurally produces after 18 to 20 hours an inflammatory exudate with cells largely of the polymorphonuclear variety (polymorphonuclear 90%, clasmatocytes or histiocytes 10%). If the animals are allowed to live three days the exudate becomes mononuclear in type. Neither of these pleural exudates after removal from the chest cavity is bactericidal for streptococci in vitro, unless it is immediately centrifugated to throw down the leukocytes, in which case, the supernatant fluids are markedly streptococcidal. These supernatant fluids retain their bactericidal action for streptococci after heating at 56 C. for $\frac{1}{2}$ hour. This power is destroyed, however, by heating at 73 C. for $\frac{1}{2}$ hour. If the supernatant fluids are left in contact for 5 hours with the cells of these exudates, they lose their bactericidal activity.

Since no explanation could be found for these interesting phenomena in terms of immunological concepts, it was thought that perhaps a study of the chemistry of the enzymes and the so-called "antienzymes" of these exudates would help in the interpretation of the facts and thus throw some light on the mechanism of immunity during the course of a streptococcus infection of the pleural cavity.

The work of Opie² on the enzymes and antienzymes of inflammatory exudates served as a starting point for our investigations. Working with pleural exudates obtained from dogs and rabbits, Opie had shown that the enzymes of the white blood cells are of two types. He demonstrated a "leucoprotease" in polymorphonuclear cells, which is active in both neutral and alkaline reactions (up to 0.2% Na_2CO_3), whereas the large mononuclears which appear toward the end of the inflammatory process contain a "lymphoprotease" which acts in a weakly acid reaction

Received for publication, Oct. 10, 1927.

* Fellow in medicine of the National Research Council.

¹ Arch. Path. & Lab. Med., 1926, 1, p. 847.

² Physiol. Rev., 1922, 2, p. 552; J. Exper. Med., 1905, 7, p. 316; *ibid.*, 1906, 8, p. 410.

(0.2% acetic acid). If the inflammatory exudate was centrifugated to throw down the cells, it was found that the supernatant fluid inhibits the activity of the proteolytic enzymes of the leukocytes. This inhibiting substance is diminished in the later stages of the inflammatory process; it is destroyed in vitro on prolonged standing, by heating to 70 C. or by rendering the reaction acid, and increase in alkalinity of the medium favors its inhibitory power. The phenomenon was observed not only with exudates obtained by injection of aleuronat, but also with the streptococcus pyogenes.

With the use of the more recent physicochemical methods we have been able to confirm and extend Opie's observations. In the following experiments, we have confined our attention to the proteolytic enzymes of the polymorphonuclear cells and studied the rôle of hydrogen-ion concentration in controlling the mechanism of enzyme-antienzyme balance.

The Preparation of Exudates.—In order to study the proteolytic activity of the polymorphonuclear leukocytes, young healthy rabbits, weighing about 1,800 to 2,000 grams, were given intrapleural injections with an aleuronat-starch mixture after the technic of Gay.³ This technic briefly is as follows: the hair above the ribs is clipped and a "button-hole" incision is made through the skin and intercostal muscles of the eighth interspace in the midaxillary line. About 5 cc. of 5% aleuronat in 3% starch solution previously sterilized and now reheated and cooled to 38 C. is injected into the pleural cavity by means of a needle having a wide bore and a blunt end. Usually both pleural cavities of a series of 6 or 8 rabbits were injected in order to get sufficient material for one experiment. The animals were killed by bleeding from the carotid artery. The pleural fluids were removed by opening the thoracic cavities.

The exudates were usually straw colored, although occasionally slightly bloody, and at times clotted in the test tube. The clots were broken up by stirring with a glass rod, the fluids were pooled and immediately centrifugated to throw down the leukocytes. The supernatant fluids were drawn off and the sedimented leukocytes washed once with physiological salt solution, then re-centrifugated and allowed to extract in distilled water for 18 hours at ice box temperature (12 C.) in the presence of a few drops of toluene.

Method of Setting Up Digestion Experiments.—Fairchild's peptone (2%) or Cooper's powdered gelatin (2%) were the substrates used in all our digestion tests. The adjustment of the P_H was made by the colorimetric method, the standard solutions having been checked with the potentiometer. Because of the retarding effects of phosphates and other salts on enzyme action⁴ no buffer salts were incorporated in any of our digestion experiments. All digestions and titrations were carried out in large test tubes about the size of Folin's digestion tubes 3×20 cm. The enzyme material obtained from 6 or 8 rabbits was diluted to about 200 cc., and 5 cc. was pipetted into each tube. All tests were done in duplicate and a third tube was set up as a "standard" color tube. Gelatin or peptone solution was also used in 5 cc. quantities. Control tubes contained 5 cc.

³ J. Infect. Dis., 1925, 36, p. 233; *ibid.*, 1923, 33, p. 338.

⁴ Falk, K. G.: Chemistry of Enzyme Action, 1925.

of water in place of gelatin or enzyme solution. As a preservative, three drops of toluene were used and the tubes tightly corked to prevent evaporation of the toluene. Digestion was allowed to proceed at 37 C. in a thermostat.

Quantitative Estimation of Proteolysis: Formol Titration Method.—Numerous methods have been published from time to time on the quantitative estimation of proteolysis. After some preliminary tests employing Brown's technic⁵ we found the formol titration of Sørensen⁶ as developed by Northrup⁷ to be the most desirable since it is accurate and requires no special apparatus. Our adaptation of the Northrup technic is briefly as follows:

Formaldehyde solution (Merck) was adjusted to P_H 9.0, using 1 drop of 0.2% phenolphthalein as an indicator. We usually added 1.0 cc. N/1 NaOH for every 20 cc. of 40% formaldehyde immediately before using. To one of the samples (containing 5 cc. of gelatin solution, 5 cc. of enzyme solution, etc.) were added 2 drops of methyl red indicator solution, 1 drop of 0.1% phenolphthalein and 1 cc. of 40% formaldehyde solution previously alkalinized as above, and then $\frac{N}{20}$ NaOH until the maximum pink color developed. This gave automatically a P_H of about 9. The preparation of this color standard, suggested by Northrup,⁷ avoids the confusion resulting from the natural colors of the samples and the destruction of color by formaldehyde.

Using one drop of methyl red as an indicator, each sample was titrated to P_H 6.0. As a guide, a standard buffer solution of P_H 6.0 was made use of. This titration was made roughly with strong alkali in the case of samples which were very acid, and then completed with $\frac{N}{20}$ NaOH. This was done in order to avoid increasing the volume which would bring into play the "water error" referred to by Harris.⁸ One cubic centimeter of alkaline formaldehyde solution was now added with 1 drop of 0.2% phenolphthalein, and the solution titrated with $\frac{N}{20}$ NaOH to match the alkaline pink standard. The amount of alkali required to bring the sample from P_H 6.0 to 9.0 was recorded as the "formol titration figure." A separate determination was made of the amount of alkali required to bring the solutions of enzyme alone, and of peptone or gelatin alone, in water, to P_H 9.0. The sum of these two values was subtracted from the average of the formol titration figures to give corrected values representing the amount of proteolysis of substrate by enzyme.

The P_H Range for the Digestion of Peptone by Rabbit Leukocytes.—Four rabbits received injections in both pleural cavities of 5 cc. aleuronat-starch mixture, and 19 hours later a total of 42 cc. of exudate was removed from the four animals. This exudate was centrifugated at once to throw down the leukocytes. The latter were washed once with physiologic salt solution and resuspended in distilled water. This was followed by extraction at 12 C. overnight. The next day the enzyme material was divided into aliquot parts and each adjusted to the desired P_H. Measured amounts of 2% Fairchild's peptone solution were also adjusted to the corresponding P_H. Into a series of 3 large test tubes were placed 5 cc. of enzyme material and 5 cc. of peptone

⁵ J. Bact., 1923, 8, p. 245.

⁶ Biochem. Ztschr., 1907, 7, p. 45; Ztsch. f. Physiol. Chem., 1909, 63, p. 27; 1910, 64, p. 120.

⁷ J. Gen. Physiol., 1926, 9, p. 767.

⁸ Harris, L. J.: Proc. Royal Society, 1923, 95, p. 440; 1923, 95, p. 500.

solution of the desired P_H . Controls were included, in which enzyme on the one hand and peptone on the other were omitted. Three drops of toluol were added to each tube and the tubes corked tightly to prevent evaporation. Incubation in the thermostat at 37 C. for 24 hours followed. Estimation of the extent of proteolysis was made by Northrop's method.⁹

As will be seen from figure 1 the leukocytes of the rabbit can split peptone over a fairly wide range, P_H 5 to 8. The optimum found after digestion for 20 hours is in the neighborhood of P_H 6.5. Similar findings were reported by Lord and Nye¹⁰ who worked with leukocytes from human pneumonic lungs and employed the Van Slyke amino-nitrogen apparatus for the estimation of proteolysis. Dernby,¹¹ working with chloroform extracts of rabbit leukocytes, found P_H 7.9 to 8.0 as the optimum for the hydrolysis of peptone. It is very probable that an extract prepared by autolyzing in chloroform for 24 hours is quite different from an aqueous extract. It is in general difficult to compare the results of different investigators because of great variation in the details of manipulation such as time of digestion, methods of extracting the enzyme material, use of buffers, method of measuring proteolysis, etc. When we tested the supernatant fluid and the whole exudate in a similar manner with peptone, the former showed its optimum digestive power at about P_H 7, and the latter at P_H 8. Using gelatin as substrate, the curves for leukocytic extract and supernatant fluid (fig. 2) presented two peaks, suggesting peptic action at P_H 3 and tryptic activity at P_H 8.

The Behavior of the Supernatant Fluid in the Presence of the Leukocytes of the Inflammatory Exudate.—With these preliminary observations before us, we set out to test the behavior of the supernatant fluid in the presence of the white blood cells of the inflammatory exudate. In one group of experiments, we observed the progressive self-digestion (autolysis) in water of various elements of the inflammatory exudate acting together and individually; in another series, under similarly controlled conditions, gelatin was added as substrate for the action of the proteolytic enzymes.

Six rabbits received injections in each pleural cavity of 5 cc. aleuronat starch mixture; 25 hours later a total of 64 cc. of exudate was removed; 48 cc. were centrifugated and the supernatant fluid was diluted up to 150 cc. with distilled water. The leukocytes derived from this fluid were suspended in a

⁹ J. Gen. Physiol., 1922, 4, p. 261.

¹⁰ Lord, F. T.: J. Exper. Med., 1919, 30, p. 379; 1922, 35, p. 153.

¹¹ Dernby, K. G.: J. Biol. Chem., 1918, 35, p. 179.

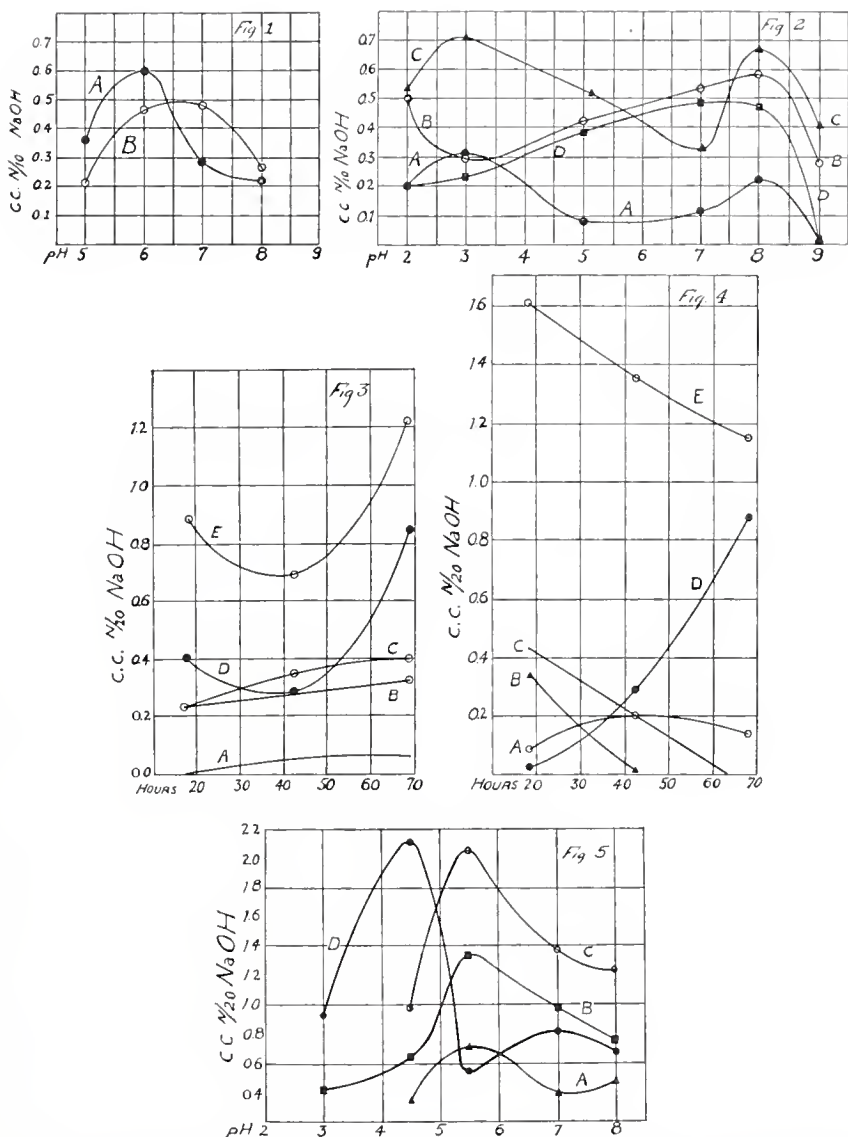


Fig. 1.—Digestion of peptone by aqueous extract of rabbit leukocytes. Ordinates give the corrected formol titration values in cc. N/10 NaOH; abscissas show the PH of digestion mixture. Each curve represents a single experiment. A—digestion for 42 hours; B—digestion for 20 hours.

Fig. 2.—Digestion of gelatin, during 44 hours at 37 C. by different constituents of pleural exudates. A—aqueous extract of leukocytes; B C—supernatant fluids; D—whole exudate. Abscissas and ordinates as in fig. 1.

Fig. 3.—The progress of autolysis of various constituents of pleural exudate. Ordinates give increase in formol titration values in cc. $\frac{N}{20}$ NaOH; abscissas give time in hours. All solutions were adjusted to PH 8. A—aqueous extract of leukocytes alone; B—supernatant fluid alone; C—sum, A + B; D—supernatant fluid and aqueous extract of leukocytes acting together; E—whole exudate (not separated).

Fig. 4.—The progress of digestion of gelatin by various constituents of pleural exudates. Ordinates give increase in formol titration values in cc. $\frac{N}{20}$ NaOH. Abscissas give time in hours. All the solutions were adjust to PH 8. A—aqueous extract of leukocytes; B—supernatant fluid; C—sum, A + B; D—supernatant fluid and extract of leukocytes together; E—whole exudate

Fig. 5.—The digestion of gelatin by various constituents of pleural exudate after 20 hours. Ordinates give formol titration values in cc. $\frac{N}{20}$ NaOH. Abscissas give PH at which digestion took place. A—aqueous extract of leukocytes; B—supernatant fluid; C—sum, A + B; D—supernatant fluid and extract of leukocytes acting together.

similar volume of water. The materials were left in the refrigerator over night. The next day the enzyme materials and substrate (gelatin) were adjusted to P_H 8.0 and tested.

In figure 3 are plotted the actual increases in the formol titration values at stated intervals, in the autolysis of various constituents of pleural exudate. The values recorded were obtained by correcting for the spontaneous increase in the enzyme solutions as well as for their initial readings. It is apparent that an aqueous extract of leukocytes autolyzes very little. The supernatant fluid, on the other hand, containing more proteins, shows active autolysis. When the two are reunited and permitted to autolyze together, there is first a greater, then a smaller, and finally a much greater amount of proteolysis than occurs in the autolysis of the constituents separately. The curve of autolysis of the whole (noncentrifuged) exudate is similar to that just described.

With gelatin as a substrate and with experimental conditions (volume, P_H and dilution) identical with those of the last experiment, we find (fig. 4) that in the case of the leukocytes (A) the curve rises progressively but begins to fall on the third day. The curves for the hydrolysis of gelatin by the supernatant fluid alone (B) or by the whole uncentrifuged exudate (E) fall rather abruptly, at the time when these enzyme solutions are undergoing active self-digestion (50 to 70 hours). The enzymes seem to digest the proteins with which they are associated in the inflammatory exudate to a greater extent than they digest gelatin. When the supernatant fluid is reunited with and tested for digestion of gelatin in the presence of enzyme from the previously sedimented leukocytes (curve D), it is surprising to note that there is during the first 24 hours a definite inhibition of proteolytic activity. This is succeeded by a rapid increase in digestion. The curves of autolysis of these solutions do not show this phenomenon.

Opie's² observation that the supernatant fluid of an inflammatory exudate contains an "antiferment" which inhibits the proteolytic action of the leukocytes is thus confirmed. But there is given here evidence that there is also a reciprocal inhibition: the leukocytes inhibit the action of the enzymes of the supernatant fluid. Moreover these inhibitory phenomena as studied in vitro are only transient and are followed by a state of mutual acceleration. The adjustment of this mechanism, as will be seen, depends on the P_H of the solutions.

The Behavior of the Socalled Antiferment Substances in the Inflammatory Exudate with Varying P_H .—Reference has already been made

to Opie's observation² that strong acids interfere with the "antiferment" action of the supernatant fluids while alkalis favor it. Since Opie had not measured the P_H of his solutions, it seemed desirable to repeat this phase of his investigations.

Eight rabbits received injections in both pleural cavities of 5 cc. of aleuronat-starch mixture, and 24 hours later the rabbits were killed by exsanguination and the pleural fluids removed. The sedimented leukocytes were suspended in 200 cc. of distilled water and allowed to extract in the refrigerator over night. The diluted supernatant fluid as well as the leukocytic extract were divided into aliquot parts and these were adjusted to P_H 3.0, 4.5, 5.5 and 8.0. Using gelatin as substrate, digestion experiments were set up in the usual manner. The period of incubation was 20 hours at 37 C.

In these experiments (fig. 5) the mutual inhibitory action of the supernatant fluid and leukocytes is evident between the limits of P_H 8 and P_H 5, and ceases abruptly on the acid side of P_H 5.0. At this acidity, the total digestion of gelatin by the supernatant fluid and leukocytes acting together is much greater than the sum of their separate actions. This would suggest a phase of mutual reinforcement following the region of inhibition of the enzymes.

DISCUSSION

The observations recorded in the present communication are strikingly similar to those of Northrop.⁹ Working with purified trypsin and employing rabbit plasma as antitrypsin, Northrop showed that the latter "protects" or rather inhibits the action of the enzyme best at P_H 8 to 9. This is due to a combination of the trypsin with the inhibiting substance, with the formation of a stable and inactive compound. On the acid side of P_H 5, trypsin does not combine with the inhibiting substance and its action is not interfered with. Since P_H 5 represents approximately the isoelectric point of the serum albumins with which, according to Cathcart¹² and Landsteiner,¹³ the so-called antitryptic substance in rabbit plasma is associated, we suggest that there is a dissociation of the enzyme-inhibitor compound on the acid side of the isoelectric point of the inhibiting protein. In order to draw any conclusions as to the relation between the behavior of the enzymes and antienzymes of the various constituents of inflammatory exudate and their bactericidal action, it would be necessary to set up parallel experiments under identical conditions. This was part of the original plan but unfortunately these experiments have had to be postponed.

The question of the relation between bacteriolysis and proteolysis is of importance not only in connection with the present problem but

¹² J. Physiol., 1904, 31, p. 497.

¹³ Centralbl. f. Bakteriol. 1, O., 1900, 27, p. 357.

in many other fundamental discussions in immunity. Jobling¹⁴ believed that there is no relationship between lysis and ferment action and Jochmann¹⁵ states that leukocytic enzyme extracts have no bactericidal action. Kantarowitz¹⁶ asserts that living bacteria, by virtue of the antiferments which they contain are very resistant to digestion by enzymes. Zinsser¹⁷ therefore concludes that in the process of phagocytosis, the bacteria must first be killed by the bactericidal substances contained in the leukocytes before they are digested by the leuko-proteases.

SUMMARY

By the use of Northrop's formol titration technic it has been possible to measure accurately the proteolysis of peptone and gelatin by various constituents of pleural exudates. These inflammatory exudates were obtained by injecting a mixture of starch and aleuronat into the pleural cavities of healthy rabbits. After removal from the chest cavity, the exudates were separated by centrifugation into supernatant fluid and sedimented leukocytes.

The leukocytes of the rabbit (extracted in distilled water) can digest peptone over a fairly wide range of hydrogen ion concentration, P_H 5 to P_H 8. The optimum is at P_H 6.5. The supernatant fluid and whole exudate show optimum digestion at P_H 7 and at P_H 8 respectively.

Using gelatin as substrate, the curves for leukocytes and supernatant fluid present two peaks, suggesting peptic activity at P_H 3 and tryptic action at P_H 8.

When the progress of digestion was followed day by day, it was found that the curves for the hydrolysis of gelatin by the supernatant fluid or by the whole exudate fall rather abruptly at the time when these enzyme solutions are undergoing active autolysis. The enzymes seem to digest the proteins with which they are associated in the inflammatory exudate rather than the gelatin.

When the supernatant fluid is reunited with, and tested in the presence of the previously sedimented leukocytes there is a definite mutual inhibition of digestive activity during the first 24 hours. This is followed by a continuous rapid rise of the curve suggesting a mutual reinforcement. The curves of autolysis of these solutions do not show this phenomenon.

¹⁴ Jobling, J. W., and Strouse, S.: Jour. Exp. Med., 1913.

¹⁵ Jochmann, G.: Leucocyten Fermente und Anti-fermente in Kolle und Wassermann. Handbuch der Pathogenen Mikroorganismen, 1913, 2, p. 1301; Friedmann, U.: Fermente und Antifermente in Festschrift zu P. Ehrlich, 1914, p. 343; Wells, H. G.: Chemical Pathology, 1924, p. 49.

¹⁶ München. med. Wehnschr., 1909, 56, p. 89.

¹⁷ Infection and Resistance, 1923.

Opie's² observation that the supernatant fluid of an inflammatory exudate contains a substance ("antiferment") which inhibits the proteolytic action of the leukocytes, is thus confirmed. Evidence is presented here that there is also a reciprocal inhibition, namely, that the leukocytes inhibit the action of the enzymes of the supernatant fluid.

This mutual inhibitory action is evident between P_H 8 and P_H 5 and ceases abruptly on the acid side of P_H 5. At this point, the total digestion of gelatin by the supernatant fluid and leukocytes acting together is much greater than the sum of their separate actions.

It is suggested that the compound, which is formed between the inhibiting substance and the enzyme, dissociates at the isoelectric point of the serum albumin, with which the so-called antitryptic substance in rabbit plasma is associated.

IS THE ANTIGENIC ACTION OF HEMOGLOBIN DUE TO GLOBIN?

LUDVIG HEKTOEN AND KAMIL SCHULHOF

From the John McCormick Institute for Infectious Diseases, Chicago

In previous articles¹ on hemoglobin precipitins we established the antigenic character of highly purified hemoglobins, thus confirming and extending the work of Ide and other early as well as recent authors. At the same time we became interested in the question, whether this property is due to the whole molecule of hemoglobin or to a simpler compound.

First of all it was found that not only oxyhemoglobin and methemoglobin, but also carboxyhemoglobin and sulphhemoglobin retained the original antigenic titer. Landsteiner and Heidelberger² found this to be true also of cyanhemoglobin. Their results differed from ours in other respects, and this paper may explain at least part of this divergence. While we observed that the precipitin titer in the ring test remained the same or almost the same after splitting the hemoglobin by acetic acid and removing the larger part of the globin by neutralization, Landsteiner and Heidelberger found that the precipitin titer after mixing antiserum with the hemoglobin solution corresponded in this experiment only to the hemoglobin content of the resulting solution as determined by Stadie's method. By this method of testing these authors observed regularly an adsorption of hemoglobin to the precipitate, but this has not occurred in the many ring or contact tests that we have made.

Since neither hematin nor globin—at least as known in 1922—was antigenic, we concluded that the antigen is either so closely adsorbed to hemoglobin that its proportion does not change after repeated treatment with aluminum cream and recrystallization, or that it forms a part of hemoglobin, which may be split off by acids. We made, as mentioned in our first paper, many attempts to separate the antigen from the colored matter, yet we were unable at that time to obtain a difference between depth of the color and the precipitin titer which would seem decisive, for instance, a four times or eight times higher titer than

Received for publication, Nov. 29, 1927.

¹ J. Infect. Dis., 1922, 31, p. 32; 1923, 33, p. 224.

² J. Exper. Med., 1923, 38, p. 561.

that which might be expected from the color of the solution. As our evidence on this point was not conclusive, we tried in various ways to split the antigen from the hemoglobin molecule. It is obvious now that progress was impeded because the compound known then as globin in reality was a denatured product, as demonstrated recently by Hill and Holden,³ who developed a method for preparation of the native compound. These authors obtained remarkable results—resynthesis of true hemoglobin from globin and hematin—which may be regarded as the first synthesis of a well defined native protein. Their method is as follows:

An equal volume of water is added to washed erythrocytes and the mixture cooled to -2°C . Enough of a similarly cooled double normal hydrochloric acid (about 0.68 cc.) is added to 5 cc. of this solution to split the hemoglobin within three or four minutes, while the mixture is being stirred. After this 5 cc. of ice-cold water are added and the solution is transferred to a 70 cc. flask, further 10 cc. of water being used to wash out the rest of the mixture. About 7 cc. of ether and 1 Gm. of purified siliceous earth (kieselguhr) are added. The flask is closed with a stopper, removed from the cold bath, wrapped in a cloth and vigorously shaken for 30 seconds, cooled again, and again shaken for 30 seconds. After cooling for one minute, the mixture is filtered in the icebox, the denatured part of the globin precipitated by adding half-normal ammonium hydroxide and the final filtrate freed from ether by a vacuum pump.

There remains only about 2% hemoglobin in the best preparations of this kind.

The reason why we failed in our attempts to obtain a larger proportion of the antigen with various adsorbents before we became acquainted with Hill and Holden's method was chiefly because we used solutions of pure hemoglobin, while Hill and Holden took advantage of the circumstance that the stroma adsorbs hematin. The other significant point of their method is the temperature of -2°C , while we worked with solutions cooled only in the ice-box.

Of the globin solutions dealt with in this paper some were prepared exactly according to Hill and Holden's method, others with additional treatment as follows: Since the denatured globin combines with hematin, we mixed the cooled acid solution of native globin with a cooled acid solution of denatured globin and neutralized the mixture with ammonium hydroxide. The filtrate thus obtained contains a smaller proportion of methemoglobin than the original.

Experiment.—A solution of native sheep globin, prepared according to Hill and Holden's method, had a protein concentration of 1:400.

³ Biochem. J., 1926, 20, p. 1326.

Its hemoglobin concentration as determined colorimetrically (Stadie) was 1:3,000. Consequently the concentration of the other proteins (chiefly globin) would be about 1:460. Precipitin serum for sheep hemoglobin gave a reaction with sheep hemoglobin at 1:50,000, with the sheep globin solution at a protein concentration of 1 to 120,000, and with sheep serum at 1:100. Now, even if all the color of the globin solution were due to native and reactive hemoglobin, the limit of precipitation would be reached when the solution was diluted 17 times ($50,000 \div 3,000 = 17$). At this point the concentration of the other proteins (globin) would be 1: (17 x 460) or 1:7,820. Since the antiserum reacted with the globin solution when diluted 300 times, that is at a protein concentration of 1:120,000, which corresponds to about 16 times the amount of globin carried by the hemoglobin present, we may conclude that much more antigen is in the globin solution than accounted for by the hemoglobin content. In fact, the result is more than expected, if globin reacts equally as strongly as hemoglobin. For the explanation of this phenomenon, it is not necessary to assume the presence of a third group. It may be due either to the inherent limits of error of the precipitin test, or globin may be a more reactive antigen. This observation may account for our former results in which the precipitin titer did not change after removal of a large portion of globin.

Other experiments gave similar results. In most of them the globin seemed to be more reactive than hemoglobin. The solutions were however, not very stable.

It seems to be established that the antigen of hemoglobin is either globin itself or a group closely connected with it. We are endeavoring at present to throw further light on this question. As even the best preparations of globin contain some hemoglobin, the production of precipitin serum by injecting rabbits with such preparation does not prove conclusively that globin is antigenic.

CONCLUSION

The antigenic group of hemoglobin forms a part of the molecule which may be split off by acids. It is probable that this antigen is globin, although the possibility of the presence of a third group is not yet excluded.

GENERAL INDEX

A

	PAGE
Abortion, streptococcus as cause of - - - - -	423
Abortus, Br., vaccines, agglutinin response to - - - - -	222
Agglutination tests for Salmonella pullorum infection - - - - -	388
Agglutinin, nonspecific, in febrile diseases - - - - -	457
Agglutinin tests with bile - - - - -	21
Agglutinins, Br. abortus - - - - -	222
ALBUS, WILLIAM R. Effect of surface tension on the growth of Escherichia coli - - - - -	211
ALEXANDER, HARRY L. See KENDALL, ARTHUR ISAAC - - - - -	137
Anaerobes from nasopharynx - - - - -	203
Anaphylaxis, effect of formaldehyde on - - - - -	137
Anaphylaxis, induction of, through intestinal mucosa - - - - -	156
Antigen, globin as, of hemoglobin - - - - -	476
Antigen, standard, for Kahn test - - - - -	111
Antigens, Salmonella pullorum, preparation of - - - - -	383
Antiproteases in pleural exudates - - - - -	467
Antitoxin, diphtheria, production of - - - - -	32
Arrows, African poisoned, bacteriology of - - - - -	51
Asthma, skin tests in - - - - -	177

B

B. aertrycke, carriers of, in wild rats - - - - -	395
B. coli, agar-dye differential medium for - - - - -	1
B. coli, continuous injection of, reaction to - - - - -	405
B. coli, effect of surface tension on growth of - - - - -	211
B. coli, injection of, renal lesions after - - - - -	448
B. enteritidis, carriers of, in wild rats - - - - -	395
B. fusiformis, biology of - - - - -	461
B. paratyphosus, mediums for isolation of - - - - -	16, 93
B. pestis caviae, carriers of, in wild rats - - - - -	395
B. sordellii from malignant edema in man - - - - -	329
B. typhosus, mediums for isolation of - - - - -	1, 16, 93
B. vulgatus, organism resembling, dissociation of - - - - -	355
B. welchii toxin, action of, on blood - - - - -	283, 289, 434
B. welchii toxin, duration of immunity to - - - - -	428
Bacilli, hemophilic - - - - -	294
Bacilli, tubercle, nonacidfast, treatment of guinea-pig with - - - - -	45
Bacteria, anaerobic, from nasopharynx - - - - -	293
Bacteria, automatic transplantation of - - - - -	276
Bacteria in spoilage of haddock - - - - -	355
Bacteria, isolation of, inoculating machine for - - - - -	190
Bacteria, metabolism of - - - - -	137, 143, 156
Bacteria of poisoned arrows - - - - -	51
Bacteria, symbiosis of - - - - -	238
Bacteria, typhoid and paratyphoid, differential mediums for - - - - -	1, 16, 93
Bacteriology of lymph - - - - -	405
Bacteriophage of thermophilic organism - - - - -	365
Bacteriophage, staphylococcus - - - - -	124
Bacterium, acidfast, from mouse - - - - -	13
BARFIELD, BLAKE. See NORTON, JOHN F. - - - - -	39
Bile, antibody tests with - - - - -	21
BLEECKER, WILLIAM L. See SCHILLING, S. J. - - - - -	222
Blood, action of B. welchii toxin with - - - - -	283, 289, 434

	PAGE
BOIKAN, WILLIAM. See PETERSEN, WILLIAM F.	405
Botulinum toxin	9, 442
BOURN, JANET M. The growth of hemophilic bacilli with certain iron salts	294
BRANHAM, SARA ELIZABETH. Anaerobic microorganisms in nasopharyngeal washings. Influenza studies XXXI	203
Brucella abortus agglutinin and agglutinins	222
BUSHNELL, L. D., and HUDSON, C. B. Complement fixation and agglutination tests for Salmonella pullorum infection	388
BUSHNELL, L. D., and HUDSON, C. B. Preparation of Salmonella pullorum antigens for complement fixation tests	383

C

CALLOW, BESSIE R. Further studies on staphylococcus bacteriophage	124
CAMPBELL, W. A. See REED, GUILFORD B.	434
Chemistry of lymph	405
Cl. botulinum, effect of Cl. sporogenes on toxin production of	442
Cl. botulinum, globulin and toxin of	9
Cl. sporogenes, effect of, on Cl. botulinum	442
Complement fixation with Salmonella pullorum antigens	383, 388
Complement, stored, degeneration of	39
CORRIGAN, MARION. Nonspecific Wassermann and agglutinin reactions with serums from patients with febrile diseases	457
Cultures, automatic transplantation of	276

D

DEGKWITZ, RUDOLPH. The etiology of measles	304
Diabetes, infectious origin of	197
Diphtheria antitoxin, production of	32
Diphtheria, scarlet fever streptococci in	377
Diplococcus, gram-positive, in protozoal infections	317
Diplococcus, green-producing, specificity of, in measles	267
Diseases, febrile, nonspecific serum reactions in	457
Dissociation of organism resembling B. vulgatus	355
DVORAK, MAYME. The effect of mosaic on globulin of potato	215

E

Edema, malignant, B. sordelli as cause of	329
Encephalitis, lethargic, relation of, to epidemic parotitis	257
Epidemic, type 4 pneumococci of	417
Epizootic, septicemic, in guinea-pigs	439
Escherichia coli, effect of surface tension on	211
ETTINGER, G. H. See REED, GUILFORD B.	439
Exudates, pleural, proteases and antiproteases of	467

F

FALK, I. S. See NORTON, JOHN F.	39
Formaldehyde, effect of, in anaphylaxis	137
Fusiform bacillus	461

G

GAMMEL, JOHN A. See TOOMEY, JOHN A.	29
GEE, A. H. Bacteria concerned in the spoilage of haddock. 2. Dissociation of an organism resembling B. vulgatus	355
Globin as antigen of hemoglobin	476
Globulin, intracellular, of Cl. botulinum	9

	PAGE
Globulin, potato, effect of mosaic on - - - - -	219
GLUNZ, KATHRYN. See SOMMER, E. WAGNER - - - - -	442
GUNDERSEN, EDVARD. Has lethargic encephalitis any relation to epidemic parotitis? - - - - -	257
GUNDERSEN, EDVARD. Is diabetes of infectious origin? - - - - -	197

H

Haddock, bacteria in spoilage of - - - - -	355
HALL, IVAN C., and SCOTT, JOSEPH P. Bacillus sordellii, a cause of malignant edema in man - - - - -	329
HALL, IVAN C., and WHITEHEAD, RICHARD W. A pharmacobacteriologic study of African poisoned arrows - - - - -	51
HEKTOEN, LUDVIG, and SCHULHOF, KAMIL. Is the antigenic action of hemoglobin due to globin? - - - - -	476
HELMHOLZ, HENRY F. The production of local renal lesions in rabbits by intravenous injection of certain strains of B. coli - - - - -	448
Hemoglobin, globin as antigen of - - - - -	476
Hemotoxins - - - - -	283, 289, 428, 434
Histamine, action of, on isolated intestine - - - - -	143
HOLMES, JANET A. See KENDALL, ARTHUR ISAAC - - - - -	137
HUDSON, C. B. See BUSHNELL, L. D. - - - - -	383, 388

I

Immunity, relation of, to tissue hydration - - - - -	177
Immunity to B. welchii toxin, duration of - - - - -	428
Immunologic relation of type 4 pneumococci - - - - -	417
Infection, protozoal, diplococcus in - - - - -	317
Infection, relation of, to diabetes - - - - -	197
Infection, Salmonella pullorum, antibody tests for - - - - -	388
Infections, skin tests in - - - - -	177
Influenza - - - - -	203
Iron salts in relation to hemophilic bacilli - - - - -	294
ISHIKAWA, MITSUTERU. Gas production by bacterial symbiosis, with special reference to the influence of nitrogenous substances - - - - -	238

J

JUNGEBLUT, CLAUS W. The optochin fastness of pneumococci - - - - -	345
--	-----

K

KAHN, R. L.; NAGLE, NATHAN, and KENDRICK, PEARL L. Studies on antigen for the Kahn test. Uniformity in sensitiveness of standard antigen - - - - -	111
Kahn test - - - - -	111, 233
KENDALL, ARTHUR ISAAC; ALEXANDER, HARRY L., and HOLMES, JANET A. The effects of formaldehyde on smooth muscle contractions in anaphylaxis. Studies in bacterial metabolism LXXXIV - - - - -	137
KENDALL, ARTHUR ISAAC, and VARNEY, PHILIP LEONARD. The physiologic action of histamine applied directly to the mucosa of the isolated surviving intestine of the guinea-pig. Studies in bacterial metabolism LXXXV - - - - -	143
KENDALL, ARTHUR ISAAC, and VARNEY, PHILIP LEONARD. Anaphylactic contraction induced through the mucosa of the isolated surviving intestine of the guinea-pig. Studies in bacterial metabolism LXXXVI - - - - -	156
KENDRICK, PEARL L. See KAHN, R. L. - - - - -	111
KOSER, STEWART A. Transmissible lysis of thermophilic organism - - - - -	365

L

	PAGE
LANGWORTHY, VIRGINIA, and MOORE, ANNA C. A study of <i>Leptospira</i>	
icterohacmorrhagiae - - - - -	70
<i>Leptospira</i> icterohaemorrhagiae - - - - -	70
<i>Leptospiras</i> from tap water - - - - -	164
LOCKE, ARTHUR; MAIN, E. R., and MILLER, F. A. The production of	
diphtheria antitoxin - - - - -	32
Lymph, bacteriology and chemistry of - - - - -	405
Lysin tests with bile - - - - -	21

M

MAIN, E. R. See LOCKE, ARTHUR - - - - -	32
MATSUMURA, K. See MEYER, K. F. - - - - -	395
McJUNKIN, F. A. Tuberculosis in guinea-pigs after treatment with tubercle	
bacilli made nonacidfast with oleic acid - - - - -	45
Measles, etiology of - - - - -	304
Measles, specificity of diplococcus of - - - - -	267
Mediums, differentiating, for typhoid groups - - - - -	1, 16, 93
Metabolism, bacterial - - - - -	137, 143, 156
MEYER, K. F., and MATSUMURA, K. The incidence of carriers of <i>B.</i>	
aertrycke (<i>B. pestis caviae</i>) and <i>B. enteritidis</i> in wild rats of San	
Francisco - - - - -	395
Microorganisms, anaerobic, from nasopharynx - - - - -	203
MILLER, F. A. See LOCKE, ARTHUR - - - - -	32
MISHULOW, LUCY; OLDENBUSCH, CAROLYN, and SCHOLL, MARIE. Potency	
of stored pertussis vaccines - - - - -	169
MOORE, ANNA. See LANGWORTHY, VIRGINIA - - - - -	70
Mosaic, effect of, on potato globulin - - - - -	215
MÜLLER, ERNST F. See PETERSEN, WILLIAM F. - - - - -	405
<i>Mycobacterium muris</i> , n. sp. - - - - -	13

N

NAGLE, NATHAN. See KAHN, R. L. - - - - -	111
Nasopharynx, anaerobes from - - - - -	203
NELSON, CASPER I. The relationship between the intracellular globulin and	
the toxin of <i>Cl. botulinum</i> - - - - -	9
NORTON, JOHN F.; BARFIELD, BLAKE, and FALK, I. S. The degeneration of	
complement on storage at several temperature - - - - -	39

O

OLDENBUSCH, CAROLYN. See MISHULOW, LUCY - - - - -	169
Optochin fastness of pneumococci - - - - -	345
ORR, J. H. See REED, GUILFORD B. - - - - -	283, 289, 434

P

Paratyphoid bacteria, mediums for differentiating - - - - -	16, 93
Parotitis, epidemic, relation of, to diabetes - - - - -	197
Parotitis, epidemic, relation of, to lethargic encephalitis - - - - -	257
Paul test for smallpox - - - - -	29
PERRY, ETHEL B. Precipitin, lysin and agglutinin tests with bile - - - - -	21
Pertussis vaccines, effect of storage on - - - - -	169
PETERSEN, WILLIAM F.; MÜLLER, ERNST, F., and BOIKAN, WILLIAM. The	
reaction of the dog to the continuous intravenous injection of <i>B. coli</i> .	
Bacteriological and chemical studies of the lymph - - - - -	405

	PAGE
PILOT, I., and WESTLUND, RUTH E. A toxin-producing hemolytic streptococcus from septicemia - - - - -	208
Pleura, exudates of, proteases of - - - - -	467
Pneumococci, optochin fastness of - - - - -	345
Pneumococci, type 4, immunologic relations of - - - - -	417
Poisons of African arrows - - - - -	51
PRATT, JOSEPHINE S. On the biology of <i>B. fusiformis</i> - - - - -	461
Precipitin reactions with globin - - - - -	476
Precipitin tests with bile - - - - -	21
Proteases of pleural exudates - - - - -	467
Protozoa, diplococcus in infections with - - - - -	317

R

RAKIETEN, MORRIS, L., and RETTGER, LEO F. Brilliant green and its use in an enrichment medium in the isolation of typhoid and paratyphoid organisms - - - - -	93
REED, GUILFORD B., and ETTINGER, G. H. An epizootic of hemorrhagic septicemia in guinea-pigs - - - - -	439
REED, GUILFORD B.; ORR, J. H., and CAMPBELL, W. A. Action of hemotoxins on oxygenated and reduced blood. 1. <i>B. welchii</i> toxin - - - - -	434
REED, GUILFORD B.; ORR, J. H., and SPENCE, C. MARION. Action of <i>B. welchii</i> toxin and other hemotoxins on erythrocytes in vivo - - - - -	283
REED, GUILFORD B., and ORR, J. H. Action of <i>B. welchii</i> toxin and other toxin and other hemotoxins on erythrocytes in vitro - - - - -	289
REED, GUILFORD B., and SPENCE, MARION. The duration of immunity to <i>B. welchii</i> toxin in rabbits - - - - -	428
REITH, ALLAN F. Streptococci as a cause of spontaneous abortion - - - - -	423
RETTGER, LEO F. See RAKIETEN, MORRIS L. - - - - -	93
RHOADS, PAUL S. The incidence of scarlet fever streptococci in throats of diphtheria patients - - - - -	377
ROBINSON, G. H. The immunologic relations of type 4 pneumococci obtained during an epidemic - - - - -	417

S

SALLE, A. J. A new agar-dye differential medium for the colon-typhoid group, with special reference to its use in water analysis - - - - -	1
Salmonella pullorum, complement fixation tests with - - - - -	383, 388
Scarlet fever streptococci in diphtheria - - - - -	377
Scarlet fever streptococci, specificity of, effect of drying on - - - - -	272
SCHILLING, S. J., and BLEECKER, WM. L. Agglutinin response to certain <i>Brucella abortus bacterins</i> - - - - -	222
SCHOLL, MARIE. See MISHULOW, LUCY - - - - -	169
SCHULHOFF, KAMIL. See HEKTOEN, LUDVIG - - - - -	476
SCOTT, JOSEPH P. See HALL, IVAN C. - - - - -	329
Septicemia, hemorrhagic, epizootic of - - - - -	439
Septicemia, toxin-producing streptococcus from - - - - -	208
SIMMONS, JAMES S. An acidfast organism isolated from a mouse. <i>Mycobacterium muris</i> , n. sp. - - - - -	13
Sinusitis, chronic, skin tests in - - - - -	177
Skin tests in asthma, sinusitis and other infections - - - - -	177
Smallpox, Paul test for - - - - -	29
Smallpox vaccines - - - - -	336
SOMMER, E. WAGNER, and GLUNZ, KATHRYN. Effect of <i>Cl. sporogenes</i> on toxin production by <i>Cl. botulinum</i> - - - - -	442
Sordellii, B., in malignant edema - - - - -	329
SPENCE, C. MARION. See REED, GUILFORD B. - - - - -	283, 428

	PAGE
Sporogenes, Cl., effect of, on Cl. botulinum - - - - -	442
Staphylococcus bacteriophage - - - - -	124
Streptococcus in relation to abortion - - - - -	423
Streptococcus, scarlet fever, effect of drying on specificity of - - - - -	272
Streptococcus, scarlet fever, in diphtheria - - - - -	377
Streptococcus, toxin-producing hemolytic, from septicemia - - - - -	208
Surface tension, effect of, on growth of Esch. coli - - - - -	211
Susceptibility, tissue hydration in relation to - - - - -	177
Symbiosis, bacterial - - - - -	238
Syphilis, experimental, Kahn test in - - - - -	233

T

Thermophile, transmissible lysis of a - - - - -	365
THOMAS, STANLEY. A comparison of reactions to dermovaccine and to neurovaccine for smallpox - - - - -	336
THOMPSON, LUTHER. Synthetic mediums in the identification of the typhoid-paratyphoid bacteria - - - - -	16
Tissue hydration in relation to susceptibility and immunity - - - - -	177
TOOMEY, JOHN A., and GAMMEL, JOHN A. The Paul test in the diagnosis of smallpox - - - - -	29
Toxin, B. welchii - - - - -	383, 289, 428, 434
Toxin, Cl. botulinum - - - - -	9, 442
Toxin-producing streptococcus from septicemia - - - - -	208
Tubercle bacilli, nonacidfast - - - - -	45
Tuberculosis after treatment with nonacidfast tubercle bacilli - - - - -	45
TUNNICLIFF, RUTH. Effect of drying on the specificity of scarlet fever streptococci - - - - -	272
TUNNICLIFF, RUTH. Further observations on the specificity of the green-producing diplococcus in measles - - - - -	267
Typhoid bacteria, mediums for differentiation of - - - - -	1, 16, 93

V

Vaccine, pertussis - - - - -	169
Vaccines, Br. abortus - - - - -	222
Vaccines, smallpox - - - - -	336
VARNEY, PHILIP L. A new spiral streak method of isolating bacteria by means of an inoculating machine - - - - -	190
VARNEY, PHILIP LEONARD. See KENDALL, ARTHUR ISAAC - - - - -	143, 156
VOGEL, W. L. A gram-positive diplococcus in human protozoal infections - - - - -	317
Vulgatus, B., organism resembling - - - - -	355

W

WALKER, JOHN E. Leptospiras from tap water - - - - -	164
WALKER, JOHN E. The Kahn test in experimental syphilis - - - - -	233
Wassermann reactions, nonspecific, in febrile diseases - - - - -	457
Water analysis, colon-typhoid differentiating medium for - - - - -	1
WEINER, W. M. Improved automatic devices for transplanting cultures - - - - -	276
WEISS, CHARLES. The proteases and antiproteases of pleural fluids - - - - -	467
Welch bacillus - - - - -	283, 289, 428, 434
WHERRY, WILLIAM B. Tissue hydration and its relation to susceptibility and immunity as shown by skin tests in asthma, chronic sinusitis and other infections - - - - -	177
WHITEHEAD, RICHARD W. See HALL, IVAN C. - - - - -	51

Form 45
614.4 V. 41 J 826

Journal of Infectious Diseases

226835

614.4 V. 41 J 826

PENNSYLVANIA STATE LIBRARY

Harrisburg 226835

In case of failure to return the books the borrower agrees to pay the original price of the same, or to replace them with other copies. The last borrower is held responsible for any mutilation.

Return this book on or before the last date stamped below.

[illegible]

